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(54) Title: DEVICE FOR SITE DIRECTED NEOVASCULARIZATION AND METHOD FOR SAME

(57) Abstract

The invention includes a device and method. The device is a site directed neovascularization device. The device includes a biocompatible support. The device also includes a biological response modifier for inducing neovascularization. The biological response modifier is adsorbed to the biocompatible support. The method is for directing *in vivo* neovascularization. The method requires adsorbing a biological response modifier for inducing neovascularization onto a biocompatible support. The step of contacting a therapeutically effective amount of the adsorbed biological response modifier to at least one selected tissue then occurs. The method then involves directing neovascular cell growth at the contacted, selected tissue for a sufficient time to obtain a vascular structure. The method of this invention is useful for developing artificial organs and other tissues including nerves in an organism, and for sampling of cells and re-implantation after genetically altering the cells to produce a desired product.

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1 **DEVICE FOR SITE DIRECTED NEOVASCULARIZATION**
2 **AND METHOD FOR SAME**

3 **BACKGROUND OF THE INVENTION**

4 **1. Field of the Invention**

5 The invention relates to a device and method for
6 directing the formation of new blood vessels and
7 artificial organs. Specifically, the invention relates
8 to a device and method for directing neovascularization
9 with a biological response modifier adsorbed onto a
10 support.

11 **2. Description of the Background Art**

12 Angiogenesis is the formation of blood vessels in
13 situ and involves the orderly migration, proliferation,
14 and differentiation of vascular cells and occurs during
15 development. Angiogenesis is an infrequent event in the

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1 adult and is associated in adults with wound and fracture
2 repair. Exceptions to this are found in the female
3 reproductive system where this process occurs in the
4 follicle during development, in the corpus luteum during
5 ovulation, and in the placenta during pregnancy. These
6 specific periods of angiogenesis are relatively brief and
7 highly regulated in contrast to the angiogenic events
8 associated with tumor growth and diabetic retinopathy.
9 The endothelial cell is considered to be the primary
10 cellular target for angiogenesis. Research efforts have
11 concentrated on the identity of polypeptide factors that
12 control endothelial cell proliferation. The
13 heparin-binding growth factor (HBGF) family of
14 polypeptides has gained general acceptance as initiators
15 of angiogenesis especially during development.

16 The gene family for producing the heparin-binding
17 growth factor family of polypeptides includes HBGF-1
18 (acidic fibroblast growth factor), HBGF-2 (basic
19 fibroblast growth factor), and three additional HBGF-like
20 structures, hst/KS, int-2, and FGF-5, each of which is
21 encoded by an oncogene. The prototype HBGF polypeptides
22 are potent inducers of endothelial cell migration and/or
23 proliferation in vitro and are known to modulate the
24 expression of endothelial cell derived proteases.
25 Further, HBGF-1 and HBGF-2 are tightly adsorbed to the
26 extracellular matrix presumably by their avid affinity
27 for the glycosaminoglycan heparin. The association
28 between the HBGF prototypes and heparin protect these
29 polypeptides from proteolytic modification. It has been
30 suggested that the extracellular matrix can be the major
31 source of HBGF-1 and HBGF-2 and activation can require
32 hydrolytic extraction from sites of attachment for
33 biological activity.

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1 Hayek, et al (1987) reported the in vivo effect of
2 fibroblast growth factor in rat kidney. (Biochem.
3 Biophys. Res. Commun. 147:876-880.) The initiation of
4 angiogenesis by the direct stimulation of endothelial
5 cell proliferation is presumed to be a result of the
6 Class I heparin-binding growth factor (HBGF-I) and the
7 Class II heparin-binding growth factor (HBGF-II). These
8 polypeptides are potent endothelial cell growth factors
9 in vitro and angiogenesis signals in vivo. These
10 polypeptides exert their biological response in vivo
11 through high affinity cell surface receptors. The HBGF-I
12 and HBGF-II share a structural similarity of 55 percent
13 and both are synthesized as polypeptides lacking an
14 apparent signal peptide sequence. Human cells which
15 express the HBGF-I mRNA transcript do not secrete the
16 polypeptide in vitro. Further, HBGF-II has been shown to
17 be associated with the extracellular matrix and heparin
18 protects HBGF-I from proteolytic modification by plasmin.

19 PCT International Publication Number WO 87/01728
20 discloses recombinant fibroblast growth factors. These
21 growth factors are examples of biological response
22 modifiers. This disclosure identifies the importance of
23 the growth factors for constructing vascular systems in
24 healing tissues. The invention of this disclosure is
25 directed to recombinant DNA sequences for encoding bovine
26 and human acidic and basic FGF and vectors bearing these
27 DNA sequences. This publication does not disclose a
28 device or method for site directed neovascularization.

29 The article, Van Brunt, et al., "Growth Factors
30 Speed Wound Healing", Biotechnology 6 (1988):25-30,
31 discloses the usefulness of growth factors in the

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1 angiogenesis of damaged tissue. This article discloses a
2 sponge implant model for wound healing in animals. The
3 sponge consists of an inert polyvinyl alcohol that is
4 implanted under the skin of the animal. Growth factor is
5 then injected directly into the sponge. The wound
6 undergoes rapid healing and an increase in blood vessels
7 occurs at the wound site. The blood vessels resulting
8 from this invention do not form complete, permanent
9 vascular structures that are directed by a support to
10 which the growth factor is adsorbed. This article does
11 not disclose a device or method for site directed
12 neovascularization.

13 U.S. Patent Number 4,699,141 to Lamberton, et al.
14 discloses a container and method for neovascularization.
15 This invention has a sponge body that is wetted
16 throughout with a solution of fibrinogen and heparin.
17 The sponge body is placed adjacent to or around a
18 noncapillary blood vessel. Capillaries then grow into
19 the sponge. The sponge can then be used as a receptacle
20 for desired cells such as pancreas cells. This patent
21 does not disclose a device or method wherein the growth
22 of blood vessels is directed in a specific direction or
23 between specific sites. Neither the heparin nor collagen
24 in this invention modify a biological response. Both the
25 heparin and collagen are substrates upon which a
26 biological response modifier acts. The capillary growth
27 developed by this invention is a result of the
28 inflammatory response of the vessel to a foreign body or
29 the sponge. The blood vessels of this invention are not
30 directed in their growth and do not form permanent
31 structures or long term structures. These blood vessels

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1 are not permanent because the fibrinogen support is
2 absorbed by the organism before maturation of the blood
3 vessels can occur.

4 The blood vessels developed by the Lamberton, et
5 al. invention are, essentially, a bundle of cells or
6 capillaries within a sponge. This invention is
7 identified as being a receptacle for "desired cells."
8 Such a receptacle is desirable for developing an
9 "artificial organ". The development of the receptacle
10 requires an undesirably long period of time of about 6
11 weeks.

12 Genetically altered or unaltered cells provide a
13 desired metabolic effect. Examples of gene transfer
14 technology to produce altered cells are provided in the
15 following three articles: Wolff, et al., "Expression of
16 Retrovirally Transduced Genes in Primary Cultures of
17 Adult Rat Hepatocytes", Proc. Natl. Acad. Sci. USA 84
18 (May 1987): 3344-3348; Ledley, et al., "Retroviral Gene
19 Transfer into Primary Hepatocytes: Implications for
20 Genetic Therapy of Liver-Specific Functions", Proc. Natl.
21 Acad. Sci. USA 84 (1987) 5335-5339; and Wilson, et al.,
22 "Retrovirus-Mediated Transduction of Adult Hepatocytes",
23 Proc. Natl. Acad. Sci. USA 85 (May 1988) 3014-3018. The
24 art is lacking a satisfactory means to transfer
25 genetically altered or unaltered cells into an organism
26 and maintain those cells permanently within that organism
27 such that the organism benefits from the desired
28 metabolic effect of the cells.

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1 The field of angiogenesis has been severely limited
2 by the absence of devices and well defined methods for
3 the selective demonstration of new blood vessel or
4 "neovessel" growth. The importance of site-directing
5 physiological neovessel formation has been long
6 recognized in medicine. The prior art has indicated the
7 possibility of such a process, but does not provide a
8 neovessel design in the form of physiological embodiments
9 for this purpose.

10 The invention is an in vivo site directed
11 neovascularization device. The device includes a
12 support. The support can be an absorbable support, a
13 non-absorbable support, or both. The device also
14 includes a biological response modifier for inducing
15 neovascularization. The biological response modifier is
16 adsorbed to support.

17 The invention also includes a method for directing
18 in vivo neovascularization. The method requires
19 adsorbing a biological response modifier for inducing
20 neovascularization onto a support. The step of
21 contacting a therapeutically effective amount of said
22 adsorbed biological response modifier to at least one
23 selected tissue then occurs. The method then involves
24 directing or culturing neovascular cell growth at the
25 contacted, selected tissue for a sufficient time to
26 obtain a vascular structure.

27 The method of this invention is useful for
28 providing artificial organs.

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1 Objects of the present invention are to provide:
2 (1) a new device for inducing site-directed
3 neovascularization; (2) a method for in vivo formation of
4 new blood vessel or a vascular bed; (3) mammalian cells
5 collected about the implanted device of the present
6 invention for multiplication, cloning, manipulation and
7 implantation thereof; (4) a vascular bed for
8 transplantation; and (5) other objects made evident from
9 the following detailed description of the invention.

10 BRIEF DESCRIPTION OF THE DRAWINGS

11 Figure 1 illustrates ECGF binding to collagen
12 supports.

13 Figure 2 illustrates the effect of implanting ECGF
14 immobilized on collagen sponges and the results thereof
15 (arrows to sponges) are shown.

16 Figure 3 illustrates the H & E histological stain
17 of sponges (IP in rat) are shown.

18 Figure 4 illustrates the site-directed gelfoam
19 implant (Sg) with GF (growth factor) between liver (left,
20 L) and spleen (right, Sp).

21 Figure 5 illustrates genetically engineered rat
22 hepatocytes recovered from collagen sponges adsorbed with
23 ECGF at 4 to 6 weeks of post-implantation.

24 Figure 6 illustrates a cross-section of a blood
25 vessel developed according to this invention.

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1 Figure 7 illustrates an angiogenic response
2 induced by HBGF-1 in situ four weeks after surgery.

3 Figure 8 illustrates the posterior portion of a
4 fiber implant containing vascular strings that are
5 generally connected to the mesentary tissue around the
6 bowel loop.

7 Figure 9 illustrates multiple vascular connections
8 between the fiber implant and mesenterial vessels and
9 vascular turbosity within the implant.

10 Figure 10 illustrates an x-ray view of the
11 multiple vascular connections of Figure 9.

12 Figure 11 illustrates a histological examination
13 of a longitudinal section that reveals the presence of
14 multiple vascular lumina surrounded by thick, collagenous
15 and muscular walls of the neovessel structure.

16 Figure 12 illustrates the vascular bundle of
17 Figure 6 at higher magnification which reveals the rich
18 collagen component of the vascular structure and
19 abundance of endothelial cell-lined capillary structures.

20 Figure 13 illustrates serum bilirubin levels of a
21 Gunn rat implanted with hepatocytes seeded onto collagen
22 (Type IV) and HBGF-1 coated PTFE fibers.

23 Figure 14A illustrates a Gortex shunt tube,
24 containing a collagen I (Gelfoam) sponge, impregnated
25 with HBGF-1, implanted onto the aorta of a rat for one
26 month, then excised and cross-sectioned.

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1 Figures 14B, 14C and 14D illustrates a Gortex
2 shunt tube containing a bundle of Gortex angel-hair
3 fibers coated with Type I collagen and impregnated with
4 HBGF-1.

5 DETAILED DESCRIPTION OF THE INVENTION

6 The invention includes both a composition or
7 "device" and a method for using that device. The device
8 is used in vivo to stimulate and direct
9 neovascularization. The neovascularization is
10 accompanied by the growth of other cellular tissue
11 including nerves. The device requires a support. The
12 support must be capable of adsorbing a biological
13 response modifier or adhering to a composition that can
14 adsorb a biological response modifier. The biological
15 response modifier is a compound that stimulates and
16 induces neovascularization. The invention further
17 includes a method for inducing neovascularization that
18 can include the development of artificial organs and/or
19 genetically engineered tissues.

20 A biological response modifier can be at least one
21 compound or agent that stimulates or facilitates vascular
22 cell growth from a tissue or organ. In other words, a
23 biological response modifier is a biochemical agent, such
24 as a growth factor, hormone, or their chimeric
25 derivative, that directly or indirectly induces a
26 transcriptional or translational cellular event. A
27 biological response modifier directly or indirectly
28 exerts an effect through a high affinity receptor. This
29 effect produces vascular cell growth. Compounds that
30 exert a direct stimulation of a receptor include
31 hormones. Compounds that provide indirect stimulation of

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1 a receptor include hormone prototypes or precursors and
2 hydrolases. Hydrolases, such as a plasminogen activator,
3 collagenase, or heparinase, initiate a biological
4 response by enzymatically activating or releasing latent,
5 stored, or zymogen precursors of direct biological
6 response modifiers.

7 Biological response modifiers desirable angiogenic
8 growth factors include a member of the group consisting
9 of HBGF-I, HBGF-II, platelet-derived growth factor
10 (PDGF), macrophage-derived growth factor (MDGF),
11 epidermal growth factor (EGF), tumor angiogenesis factor
12 (TAF), endothelial cell growth factor (ECGF), fibroblast
13 growth factor (FGF), hypothalamus-derived growth factor
14 (HDGF), retina-derived growth factor (RDGF), and mixtures
15 thereof. The preferred embodiment of the invention uses
16 HBGF-I. Desirable hydrolases include a member selected
17 from the group consisting of heparinase, collagenase,
18 plasmin, a plasminogen activator, thrombin, heparatinase,
19 and mixtures thereof.

20 Hormones such as the growth factors are
21 particularly desirable for use in this invention as
22 biological response modifiers. Hormones specifically
23 elicit cell growth and differentiation. The use of
24 hormones as biological response modifiers cause
25 neovascularization to rapidly occur and to form a
26 complete vascular structure. The resulting blood vessel
27 stimulated by hormones is more than just a mass of cells
28 in that it has a tubular cavity and connective tissue
29 between its cells. The resulting blood vessel produced

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1 from the use of hormones is complete within itself and
2 can be excised and transplanted into another portion of
3 the body. The other biological response modifiers
4 produce similar results, but do not provide as rapid a
5 growth as hormones and, in particular, the HBGF-I and
6 HBGF-II hormones.

7 The invention includes a biocompatible support to
8 which the biological response modifier is adsorbed.
9 The support can be either or both an absorbable or
10 non-absorbable biocompatible matrix. The support must be
11 implantable into an organism and is, desirably, rigid and
12 strong enough to be transplantable after
13 neovascularization has occurred. The biocompatible
14 support must have the rigidity and strength to support
15 neovascularization. Examples of absorbable supports
16 include a member selected from the group consisting of
17 collagen Type I, known commercially by the trade name
18 "Gelfoam", laminins, fibronectins, gelatins,
19 glycosaminoglycans, glycolipids, proteolipids,
20 mucopolysaccharides, glycoproteins, polypeptides, and
21 mixtures thereof. Examples of non-absorbable matrices
22 include members of the group consisting of nylon, rayon,
23 dacron, polypropylene, polyethylene, expanded PTFE,
24 cross-linked collagen Type IV, and mixtures thereof. It
25 is desirable that a selected support contain
26 extracellular matrix protein to provide or to facilitate
27 the adsorption of the biological response modifier to the
28 biocompatible support.

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1 An extracellular matrix protein can be the
2 material from which the biocompatible support is formed
3 or a component added to the biocompatible support to
4 fully provide or, alternatively, facilitate the
5 adsorption of the biological response modifier to the
6 biocompatible support. An extracellular matrix protein
7 component can include a pure or mixed composition of
8 proteins or polypeptides. The proteins and polypeptides
9 can be either natural or synthetic. The extracellular
10 matrix protein component is desirably derived from
11 extracellular structural molecules. These extracellular
12 structural molecules include a member selected from the
13 group consisting of collagens, laminins, fibronectins,
14 gelatins, glycosaminoglycans, glycoproteins,
15 proteoglycans, and mixtures thereof.

16 Expanded polytetrafluoroethylene (PTFE) has been
17 found to be most suitable non-absorbable support for this
18 invention. This support provides the following
19 benefits. PTFE has a general lack of an inflammatory
20 response which is the basis for the current acceptance of
21 PTFE in the surgical community. PTFE can be coated
22 conveniently with various components of the extra

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1 cellular matrix which can adsorb a biological response
2 modifier. Biologically active HBGF-1 and HBGF-2 can be
3 immobilized to collagen-coated PTFE by previously
4 established methods. PTFE polymers are routinely
5 engineered to various specifications to meet a multitude
6 of required configurations.

7 The configuration of the non-absorbable PTFE is a
8 more critical aspect of the long-term implant model. All
9 multicellular organisms utilize a three-dimensional
10 architecture of branching fiber networks to solve the
11 problem of increasing surface area in a given volume.
12 Seeding of such a network with HBGF polypeptides before
13 implantation allows for high localized concentrations of
14 the mitogen. Non-woven multifilament angel-hair fibers
15 of expanded PTFE are commercially available from W.L.
16 Gore and Associates, Inc., Flagstaff, Arizona. These
17 fibers allow sufficient organized surface area for
18 infiltrating cells to be exposed to the environment of
19 the host. This permits the free exchange of nutrients
20 and toxic waste to occur while neovascularization
21 processes occur. Furthermore, cell shape as determined
22 by cytoskeletal components and attachment to a specific
23 matrix generally is regarded to play a significant role
24 in both cell proliferation and differentiation.

25 A support can be provided for use in this
26 invention in any desired shape and size. A support as
27 small as one 1mm^2 is suitable to provide a base for
28 neovascularization. Desirable shapes for a support can

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1 be a strip, a sponge, or a tube. Supports are desirably
2 capable of being secured within an organism. Suitable
3 means for securing a support can include a staple,
4 biocompatible glue, or other surgical procedures such as
5 suturing or tying the support to a tissue.

6 A desirable support is obtained by filling a tube
7 or sleeve of expanded PTFE with expanded PTFE fibers or
8 "angel hair". Supports formed from tubes or sleeves
9 provide a pouch for an artificial organ. The tubular
10 form of the support and the bundle of fibers within the
11 tube are particularly desirable for directing
12 neovascularization. Such embodiments can be receptacles
13 for implanted cells when the invention is used to provide
14 an artificial organ.

15 The most effective concentrations for a biological
16 response modifier can be any concentration that elicits a
17 growth response from the target cells, but is not toxic
18 to those cells. Effective or therapeutic concentrations
19 of angiogenetic growth factors are between about 1 to
20 about 10 nanograms per cubic millimeter of a support. A
21 support for this calculation includes both the absorbable
22 support and the non-absorbable support.

23 A support is provided in an amount suitable to
24 establish the length and width of the desired blood
25 vessel. For example, if a blood vessel is desired between
26 two tissues and there exists a distance between those two
27 tissues, then a corresponding length of support is

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1 implanted into the organism to provide the approximate
2 length and width of this desired blood vessel. The
3 amount of the biological response modifier is then
4 adapted to the amount of support required to form this
5 basic structure.

6 The invention can be practiced without a
7 non-absorbable support. For example, a complex with
8 gelatin, HBGF-1, or HBGF-2 is capable of inducing
9 neovascularization in vivo at polypeptide concentrations
10 consistent with the demonstration of this biological
11 activity in vivo. This neovascular response is capable
12 of sustaining induced site-specific neovessel formation
13 for up to four weeks in the neck and peritoneal cavity of
14 the rat. However, the device of this invention without a
15 support has limited utility for the induction of
16 long-term neovessels. This is because the
17 three-dimensional architecture of the collagen sponges is
18 ultimately disrupted by a reabsorption process that
19 occurs within three to four weeks after implantation.
20 Nonabsorbable solid polymeric supports of well-defined
21 specifications and containing bonded components of
22 extracellular matrices induced the expression of
23 long-term stable neovessels in vivo. An example of such
24 an embodiment is a nonabsorbable support bonded with both
25 collagens Type I and Type IV and having both HBGF-1 and
26 HBGF-2 attached to the collagens.

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1 A neovascularization device can also be seeded
2 with desired cells prior to or subsequent to implantation
3 in a host. In a preferred embodiment, such cells are
4 mammalian cells and express a protein capable of
5 performing a particular function. The cells can be
6 genetically engineered cells capable of expressing a
7 heterologous protein. Alternatively, the cells can be
8 naturally occurring cells capable of providing a desired
9 function or functions such as hepatocytes.

10 Desirable embodiments of the invention have cells
11 seeded in or on the neovascularization device which are
12 genetically engineered to express at least one
13 heterologous protein. Such a protein is preferably a
14 therapeutic agent. The expressed protein may or may not
15 be secreted from the genetically engineered cells.

16 The genetically engineered cells used with this
17 invention are transformed with at least one gene that
18 encodes for the desired heterologous protein. The cells
19 are transformed with a suitable vector or expression
20 vehicle which includes the desired gene. The vector can
21 also include a promoter for expression in the host
22 cells. In mammalian cells, the promoter for expression
23 can be SV 40, LTR, metallothionein, PGK, CMV, ADA, TK, or
24 others. The vector can also include a suitable signal
25 sequence or sequences for secreting the therapeutic agent
26 from the cells. The selection of a suitable promoter is
27 deemed to be within the skill of the art.

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1 The vector or expression vehicle is preferably a
2 viral vector and in particular a retroviral vector.
3 Representative examples of suitable viral vectors, which
4 can be modified to include a gene for a therapeutic
5 agent, include Harvey Sarcoma virus, ROUS Sarcoma virus,
6 MPSV, Moloney murine leukemia virus, DNA viruses such as
7 adenovirus and others. Alternatively, the expression
8 vehicle can be a plasmid. Transformation can be
9 accomplished by liposome fusion, calcium phosphate or
10 dextran sulfate transfection, electroporation,
11 lipofection, tungsten particles, or other procedures.
12 The selection of a suitable vehicle for transformation is
13 deemed to be within the scope of those skilled in the
14 art.

15 When a retroviral vector is employed as the
16 expression vehicle for transforming cells, steps should
17 be taken to eliminate and/or minimize the chances for
18 replication of the virus. Various procedures are known
19 in the art for providing helper cells which produce viral
20 vector particles that are essentially free of replicating
21 virus. Examples of such procedures are found in
22 Markowitz, et al., "A Safe Packaging Line for Gene
23 Transfer; Separating Viral Genes on Two Different
24 Plasmids", Journal of Virology 62(4) (April
25 1988):1120-1124; Watanabe, et al., "Construction of a
26 Helper Cell Line for Avian Reticuloendotheliosis Virus
27 Cloning Vectors", Molecular and Cellular Biology 3(12)
28 (Dec. 1983):2241-2249; Danos, et al., "Safe and Efficient
29 Generation of Recombinant Retroviruses with Amphotropic

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1 and Ecotropic Host Range", Proc. Natl. Acad. Sci. 85
2 (Sept. 1988):6460-6464; and Bosselman, et al.,
3 "Replication-Defective Chimeric Helper Provirus and
4 Factors Affecting Generation of Competent Virus;
5 Expression of Moloney Murine Leukemia Virus Structural
6 Genes via the Metallothionein Promoter", Molecular and
7 Cellular Biology (5) (May 1987):1797-1806 disclose
8 procedures for producing a helper cell which minimizes
9 the chances for producing a viral particle that includes
10 replicating virus. This procedure and other procedures
11 can be employed for genetically engineering cells by use
12 of a retroviral vector. In addition to the promotor and
13 the gene for the therapeutic agent, other material can be
14 included in the vector. This material can include a
15 selection gene such as a neomycin resistance gene, a
16 sequence for enhancing expression, or other materials.

17 Genetically engineered mammalian cells can be
18 implanted in a mammal by use of a neovascularization
19 device. These genetically engineered cells are desirably
20 implanted into a mammal of the same species. In a
21 preferred embodiment, the genetically engineered
22 mammalian cells are cells originally derived from a
23 patient, genetically engineered to include a gene for at
24 least one therapeutic agent, and implanted into the
25 patient from which they were derived by use of a
26 neovascularization device in accordance with the
27 invention. These autologous genetically engineered cells
28 then provide "gene therapy" by in vivo production of the
29 therapeutic agent for treatment of the patient.

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1 The genetically engineered cells can be engineered
2 such that the therapeutic agent is secreted from the
3 cells in order to exert its effect upon cells and tissues
4 either in the immediate vicinity or in more distal
5 locations. Alternatively, the therapeutic agent, if it
6 is not secreted from the engineered cells, exerts its
7 effect within or on the engineered cells and can cause
8 the metabolism of substances that diffuse into or onto
9 the cells. Examples of such therapeutic agents include
10 adenosine deaminase (ADA) that functions within the cell
11 to inactivate adenosine, a toxic metabolite that
12 accumulates in severe combined immunodeficiency syndrome,
13 or phenylalanine hydroxylase that functions within a cell
14 to inactivate phenylalanine, a toxic metabolite in
15 phenylketonuria.

16 The genetically engineered cells used with this
17 invention are transformed with a gene for at least one
18 heterologous protein. This protein is preferably a
19 therapeutic agent. The term "therapeutic agent" is used
20 in its broadest sense and means any agent or material
21 which has a desired or beneficial effect on the host.
22 The therapeutic agent can be more than one type of
23 protein. Desirable proteins include CD-4, Factor VIII,
24 Factor IX, von Willebrand Factor, TPA, urokinase,
25 hirudin, the interferons, tumor necrosis factor, the
26 interleukins, hemotopoietic growth factors including
27 G-CSF, GM-CSF, IL3, erythropoietin, antibodies,

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1 glucocerebrosidase, ADA, phenylalanine hydroxylase, human
2 growth hormone, insulin and others. The selection of a
3 suitable gene is deemed to be within the scope of those
4 skilled in the art. Mixtures of cell types can also be
5 used with this invention such as genetically engineered
6 smooth muscle cells, fibroblasts, glial cells,
7 keratinocytes, or others.

8 The effect in genetically engineered cells when
9 used in gene therapy, can be controlled by the selection
10 of high producing clonal populations and/or the use of
11 vectors with enhanced expression. This can provide, in
12 vivo, therapeutically effective amounts of a desired
13 therapeutic agent for treating a patient. In determining
14 the number of cells to be implanted, factors such as the
15 half life of the therapeutic agent, volume of the
16 vascular system, production rate of the therapeutic agent
17 by cells, and the desired dosage level are considered.
18 The selection of such vectors and cells is dependent on
19 the therapeutic agent and is within the scope of those
20 skilled in the art.

21 The neovascularization device of the invention can
22 also be employed to obtain cells from a host by
23 implanting the device in a host and after a period of
24 time removing the implanted neovascularization device
25 from the host for recovery of cells which have been
26 collected on the device. Such cells can be
27 differentiated and used for a variety of purposes. For

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1 example, this procedure can provide a source of
2 autologous cells for genetic engineering and subsequent
3 return to the host as genetically engineered cells for
4 expression of a protein. Cells collected in this manner
5 can be genetically engineered and then returned to the
6 host to provide an artificial organ.

7 The process for directing neovascularization first
8 involves preparing the device of this invention as
9 described above. The device is prepared by adsorbing a
10 biological response modifier, that is suitable for
11 inducing neovascularization, onto a support. The
12 biological response modifier must be present on the
13 support in such a concentration as to be therapeutically
14 effective for eliciting cell growth. The adsorbed
15 biological response modifier is then contacted to at
16 least one selected tissue. Typically, the device is
17 connected to at least two separate sites between which a
18 blood vessel is desired. These two sites can be the same
19 or separate tissues or organs. The method then involves
20 culturing neovascular cell growth at or from the
21 contacted tissue. Culturing of the contacted cells must
22 occur for a sufficient time to allow or enable
23 neovascularization and the vascular structure to form.

24 Figure 1 demonstrates that ECGF binds to collagen
25 supports. This is shown by an elution profile of HBGF-1
26 (ECGF) from collagen type IV-Sepharose and
27 gelatin-Sepharose columns. Collagen Type IV-Sepharose
28 and The gelatin-Sepharose (1 ml) were packed in a column

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1 and washed with 5 mls of 2M NaCl in 50mM Tris HCl, pH
2 7.4, followed by an exhaustive wash with 50mM Tris HCl,
3 pH 7.4 (adsorbtion buffer; AB). The Gelatin-Sepharose
4 was from Pharmacia. Bovine collagen-Type IV-Sepharose
5 was obtained from Sigma Chemical Company, St. Louis, MO.
6 and (^{125}I)-HBGF-1 was prepared as previously described.
7 (^{125}I)-HBGF-1 (approximately 5×10^5 cpm) in absorption
8 buffer was added to the column in a volume of
9 approximately 0.1 ml and the column washed with
10 absorption buffer. Elution of column-associated
11 (^{125}I)-HBGF-I was achieved with 1.5M NaCl in absorption
12 buffer or 50 units of heparin (Upjohn, Kalamazoo, MI) in
13 absorbtion buffer. The NaCl-eluted column was
14 regenerated with an absorption buffer wash and the
15 heparin-eluted column was regenerated by consecutive
16 washes with 1.5M NaCl in absorption buffer followed by
17 another wash with absorbtion buffer. The matrix affinity
18 procedures were performed at room temperature (about 22°C
19 to 25°C).

20 Figure 2 demonstrates that ECGF binds to collagen
21 supports. The adsorbed factor was implanted in various
22 anatomical sites to demonstrate the practicality of using
23 growth factor-adsorbed implants to stimulate neovessel
24 formation and the growth of vascular beds in areas of
25 interest. The effect of implanting ECGF immobilized on
26 collagen sponges and the results thereof (arrows to
27 sponges) are shown:

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- 1 A. Neck, 2 weeks, no ECGF;
- 2 B. Neck, 2 weeks, plus ECGF;
- 3 C. IP, 2 weeks, no ECGF;
- 4 D. IP, 2 weeks, plus ECGF;
- 5 E. IP, 2 weeks, plus ECGF site-directed; and
- 6 F. IP, 2 weeks, plus ECGF implantation in
- 7 omentum.

8 Figure 3 demonstrates that the device of this
9 invention induces significant angiogenesis in situ.
10 These implants were removed at various times for
11 examination by common methods of histology in order to
12 determine the microscopic nature of these dynamics. The
13 following abbreviations are used: Sg represents "sponge
14 (C-1)"; Sp represents "spleen"; L represents "liver"; and
15 BV represents "blood vessel (aorta)". H & E histological
16 stain of sponges (IP in rat) are shown:

- 17 A. sponge--two weeks, IP, without ECGF;
- 18 B. sponge--one week, IP, plus ECGF;
- 19 C. sponge--two weeks, IP, plus ECGF;
- 20 D. sponge glued to liver, 2 weeks, plus ECGF;
- 21 E. sponge glued to spleen, 2 weeks, plus ECGF;
- 22 and
- 23 F. sponge wrapped around aorta, 2 weeks, plus
- 24 ECGF.

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1 Figure 4 demonstrates that ECGF induces
2 significant and stable angiogenic response in situ by the
3 recruitment of appropriate cell types as shown in Figures
4 2 and 3. Implants were established to create
5 site-directed bridges between a large variety of organs,
6 vessels, tissues and the like. Illustrated are the
7 site-directed Gelfoam implant (Sg) with growth factor
8 (GF) between liver (left, L) and spleen (right, Sp).

9 Figure 5 demonstrates that the device of this
10 invention serves to create neovessels independent of the
11 implantation site in situ. The device has an ability to
12 serve as a recruitment vehicle for mammalian cells in
13 general and as a vehicle to maintain the viability and
14 physiological environment for and of the implanted and
15 transplanted cells. Genetically engineered rat
16 hepatocytes recovered from collagen sponges adsorbed with
17 ECGF after 4 to 6 weeks post-implantation are shown.
18 Hepatocytes were removed to determine their viability.

19 Figure 5A shows the results with no growth
20 factor. Note that in Figure 5A few cells appear to be
21 unhealthy and there is no proliferation or growth of
22 survivor cells. Figure 5B shows the results with growth
23 factor. Note that in Figure 5B healthy viable cells are
24 accompanied by significant proliferation.

25 The device and method of this invention can
26 provide angiogenesis and neovascularization from one or
27 more sites on a single tissue or different tissues. The
28 development of a blood vessel from a single site of one

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1 tissue, such as an artery, provides a vessel that can be
2 transplanted or that can be used as an artificial organ.
3 The development of a blood vessel between two or more
4 sites located on the same or different tissues provides
5 improved circulation between the sites.

6 Figure 6 illustrates a cross section of a blood
7 vascular structure developed by the device and method of
8 this invention. This figure demonstrates that the blood
9 vessels developed by this invention are not merely a
10 bundle of vascular cells growing in an undirected
11 manner. The blood vessel 1 contains endothelial cells 2,
12 mesothelial cells 3, pericytes 4, smooth muscle cells 5,
13 fibroblasts 6, and neuronal-like cells 7. The cross
14 section of the blood vessel 1 demonstrates the formation
15 of capillary-like structures 8, arteries 9, and vein-like
16 structures 10. This development of a complete vascular
17 structure provides a rigid vessel that remains
18 permanently in the organism and that can be transplanted
19 within this organism.

20 A method of this invention can be used to provide
21 an artificial organ by first directing the growth and
22 development of a blood vessel from a tissue. The
23 developed blood vessel is then injected or seeded with
24 cells from a selected tissue or organ. The injected
25 cells can be genetically altered before being seeded into
26 the blood vessel. The seeded cells can provide a desired
27 metabolic effect. These metabolic effects can include

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1 hepatic functions such as bilirubin metabolism and
2 pancreatic functions such as insulin production. Other
3 metabolic functions can be provided by cells containing
4 one or more hormone producing genes. Artificial organs
5 developed according to this invention can provide desired
6 functions without being subject to a response from the
7 organism's immune system.

8

EXAMPLE 1

9 Example 1 demonstrates various embodiments of the
10 device or composition of the invention and the method by
11 which the device is produced. This example uses HBGF-I
12 with a radioactive iodine marker. In therapeutic use,
13 the radioactive marker would not be present. Example 1
14 is as follows.

15 Gelatin-Sepharose and collagen Type IV-Sepharose
16 were examined for the ability to absorb (^{125}I) -HBGF-1.
17 Figures 1C and G show that the majority or approximately
18 80 percent of the (^{125}I) -HBGF-1 binds to immobilized
19 gelatin and collagen Type IV and can be eluted with 1.5M
20 NaCl. Adsorbed (^{125}I) -HBGF-1 can also be eluted
21 with 0.5M NaCl (data not shown). Denaturation of
22 (^{125}I) -HBGF-1 by heating at 90°C for 1 minute
23 significantly reduces the ability of the polypeptide to
24 bind to immobilized gelatin and collagen Type IV by
25 inactivation of the binding domain within the HBGF-1
26 polypeptide structure.

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1 The (^{125}I)-HBGF-1 adsorbed to immobilized gelatin
2 and collagen Type IV can also be eluted with heparin as
3 shown in Figures 1A and E. Approximately 20% of the
4 growth factor, which remains bound after heparin elution,
5 can be eluted with 1.5M NaCl.

6 Pretreatment of the gelatin and collagen Type IV
7 matrix with 50 units of heparin significantly reduces the
8 ability of either matrix to absorb (^{125}I)-HBGF-1 as shown
9 in Figures 1B and F. Regeneration of either matrix with
10 a 1.5M NaCl wash permits (^{125}I)-HBGF-1 adsorption.

11 Bovine serum albumin at 1mg per ml and human
12 fibronectin at 1mg per ml do not significantly elute
13 (¹²⁵I)-HBGF-1 absorbed to either matrix as shown in
14 Figures 1D and H.

15 EXAMPLE 2

Example 2 demonstrates the method for implantation of the device of this invention and for eliciting neovascularization. The use of immobilized gelatin with HBGF-I represents the preferred embodiment of the invented method. Example 2 is as follows.

21 Example 2 demonstrates that HBGF-I binds to both
22 immobilized gelatin and to collagen Type IV. It is shown
23 that HBGF-I, adsorbed to gelatin sponges, promotes

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1 angiogenesis in the rat at concentrations of the growth
2 factor which are consistent with the growth factor's
3 activity as an endothelial cell mitogen in vitro. This
4 concentration is about 10^{-3} times lower than the
5 concentration used in vitro in the art.

6 The abdomen of an anesthetized male rats weighing
7 250 grams was washed with 20 percent volume to weight
8 (v/w) ethanol and an incision was made into the abdominal
9 cavity wall to expose the abdominal cavity. Gelfoam,
10 manufactured by Upjohn, Kalamazoo, Michigan, was cut into
11 strips of approximately 5 by 20mm. The sponge was
12 cemented to the distal area of the abdominal aorta with
13 n-butylcyanoacrylate. A bridge was created with the free
14 end of the sponge when the free end was cemented to
15 another tissue. In the studies that were conducted to
16 provide these examples, the following tissues were
17 actually contacted by the device. These tissues were
18 other organs including the liver, kidney, and spleen, the
19 abdominal cavity, and other macro and micro vessels.
20 Various concentrations of HBGF-1 from about 1 to about 10
21 ng per mm^3 were adsorbed to sponges for these studies.
22 The surgical opening was closed with a staple gun. The
23 animals were fed a normal diet and the incision was
24 opened 1 week after surgery. The collagen sponge was
25 surgically extracted, grossly examined for blood vessel
26 formation and the sponge prepared for histological
27 examination.

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1 It is known that HBGF-1 binds to immobilized
2 gelatin and collagen Type IV, therefore, the possibility
3 was evaluated that commercial gelatin sponges sold by the
4 tradename "Gelfoam" adsorbed with HBGF-1 could be
5 utilized as a method for inducing angiogenesis in situ.
6 Survival surgery was performed on the rat in order to
7 implant gelatin sponges which were treated with HBGF-1.
8 HBGF-1-adsorbed Gelfoam was independently placed in the
9 neck and peritoneal cavities in the rat. A significant
10 angiogenic response was observed in situ one week after
11 surgery with 1ng HBGF-1 per mm² (Figure 2). Blood
12 vessels, which migrated away from the tissue site of
13 implantation, were observed macroscopically to be
14 exclusively within the gelatin sponge. Control sponges
15 without HBGF-1 and sponges adsorbed with HBGF-1 and
16 heparin did not induce neovascularization after one week
17 in vivo. The latter is consistent with the ability of
18 heparin to prevent HBGF-1 adsorption to immobilized
19 gelatin and collagen-Type IV. A titration curve with
20 various concentrations of HBGF-1 was performed using this
21 procedure and results similar to Figure 1 was observed
22 with 1 to 10ng HBGF-1 per mm³ of sponge (data not
23 shown). Histological examination (Figure 3) of the
24 sponge removed after one week in situ revealed new blood
25 vessel growth within the sponge.

26 Since HBGF-1-adsorbed Gelfoam alone (without more)
27 is an efficient inducer of angiogenesis from the serosa.
28 The ability of immobilized HBGF-1-adsorbed implants to
29 induce and sustain the process of neovascularization
30 within the peritoneal cavity was assessed. Separate

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1 surgical implants were cemented as strips of Gelfoam to
2 the abdominal aorta in the rat creating a bridge between
3 this site and either the kidney, spleen, liver, or
4 abdominal wall (Figure 4). After two weeks in vivo, the
5 implants were examined for the extent of angiogenesis.
6 Bidirectional formation of new blood vessels along the
7 HBGF-1-adsorbed gelatin sponge from the liver and aorta
8 was observed. Similar bidirectional results were
9 observed with implants cemented from the aorta to either
10 the kidney, spleen, or abdominal wall (data not shown).
11 Histological examination of these implants yielded
12 results identical to those observed in Figure 3.

13 Induced neovascularization within the peritoneal
14 cavity was also shown to sustain the proliferative
15 potential of a genetically engineered rat hepatocyte cell
16 strain simultaneously implanted with the HBGF-1-adsorbed
17 Gelfoam (Figure 5). Hepatocytes were grown to high
18 density (10^8 cells) on a Gelfoam sponge. Prior to
19 surgical implantation, 10ng of HBGF-1 per mm^3 of sponge
20 was added. Control sponges did not contain any adsorbed
21 HBGF-1. Separate surgical implants were cemented as a
22 bridge between the liver and the spleen and allowed to
23 remain in situ for four to six weeks. At this time, the
24 implants were removed, digested with either trypsin or
25 collagenase to recover implanted cells which were
26 maintained in tissue culture. Cells which were recovered
27 from HBGF-1-adsorbed Gelfoam sponges were able to
28 proliferate in vitro under selective pressure which

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1 reflected genetic disposition (Figure 5B). In contrast,
2 the cells recovered from control Gelfoam sponges
3 displayed a loss of proliferative potential (Figure 5A).
4 Histological examination of sponges containing the cells
5 revealed that HBGF-1 also induced a response similar to
6 Figures 3 and 4.

7 In accordance with the device and method of the
8 present invention, angiogenesis and neovascularization
9 has been achieved between various tissues and organs as
10 demonstrated by Figures 2 through 5. Neovascularization
11 has been similarly accomplished between the following
12 loci (data not shown): liver to spleen; liver to kidney;
13 spleen to kidney; liver to aorta; liver to vena cava;
14 liver to omentum (omentum, containing pancreatic tissue);
15 aorta/to vena cava; spleen to aorta; spleen to vena cava;
16 spleen to omentum kidney to aorta; kidney to vena cava;
17 kidney to omentum; omentum to aorta; and omentum to vena
18 cava.

19 EXAMPLE 3 AND COMPARATIVE EXAMPLE A

20 Example 3 demonstrates the device of the invention
21 having a non-absorbable support. The experiments
22 performed to derive this example were conducted with
23 either Type I or Type IV collagen and involved
24 implantation onto the liver or the spleen of a rat.

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1 Comparative Example A demonstrates that the use of
2 the same materials and procedures of Example 3 without
3 HBGF-1 did not induce neovascularization.

4 HBGF-1 adsorbed, collagen-coated (Type I or IV)
5 expanded PTFE fibers were surgically implanted in the
6 peritoneal cavity (onto the liver or the spleen) of the
7 rat. A significant angiogenic response was specifically
8 induced by HBGF-1 in situ and the results four weeks
9 after surgery are shown in Figure 7. Blood vessels,
10 which have migrated from the tissue site of implantation,
11 could be observed macroscopically within and around the
12 implanted fibers. The anterior portion of the fiber
13 implant, which was attached to the liver, exhibited
14 substantial neovessel growth from the liver into the
15 interior of the implant (Figure 7). Further examination
16 revealed that the posterior portion of the fiber implant
17 (attached to a specific organ) or regions in the vicinity
18 of the implant contained vascular "strings" which were
19 generally connected to the mesentary tissue around the
20 bowel loop (Figure 8). It was also possible to induce
21 and sustain long-term bi-directional neovessel formation
22 between the liver and spleen by the implantation of
23 separate HBGF-1-treated fibers on each organ. The
24 ability of HBGF-1 adsorbed implants to maintain the
25 neovessel structures within the peritoneum is evidenced
26 by these highly vascular bridges. Control fibers of
27 Comparative Example A did not induce neovascularization
28 even after six months following surgical implantation.
29 Titrations with various concentrations of HBGF-1 were
30 performed using this procedure. Similar results were

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1 obtained with HBGF-1 at concentrations between 1 to 100
2 ng/mm³ of fiber surface area. The concentration of
3 HBGF-1 required to induce an angiogenic response in the
4 fiber implant model is consistent with the results
5 obtained with the Gelfoam implant model and the mitogenic
6 activity of the polypeptide in vitro.

7

EXAMPLE 4

8 Example 4 demonstrates that the blood vessel
9 produced in Example 3 displayed a large organized solid
10 matrix including a network of neovessel formations.

11 Two months following surgical placement of the
12 HBGF-1-treated implant on the spleen of a rat, the
13 abdominal organs were perfused and fixed (formaline)
14 using a catheter placed in the lower thoracic aorta.
15 Subsequently, the abdominal organs were perfused with a
16 radio-opaque silicone rubber dye sold by the trademark,
17 Microfil, followed by soft X-ray analysis (magnification
18 27KV). Multiple vascular connections between the fiber
19 implant and mesenterial vessels were observed as well as
20 a vascular turbidity within the implant which is typical
21 for new vessel formation (Figure 9). Histological
22 examination of the implant itself displayed a large
23 organized solid matrix containing a network of neovessel
24 formations interdigitated with different cell types,
25 which is consistent with results previously obtained with
26 the short-term HBGF-1-treated Gelfoam implant model.
27 X-ray analysis of the long-term fiber implant as shown in
28 Figure 10 has confirmed that neovessel formation within
29 the fiber network has become integrated with the vascular
30 tree of the host, primarily through the bridges

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1 ("strings") of richly vascular tissue (Figures 7 and 8).
2 Histological examination of the longitudinal section
3 through a typical vascular connection revealed the
4 presence of multiple vascular lumina surrounded by thick,
5 collagenous and muscular walls of the neovessel structure
6 (Figure 11). Cross-sectional analysis through these
7 vascular connections further related the presence of a
8 monolayer of mesothelial cells surrounding a large
9 vascular lumina in the central portion, encompassed by
10 prominent endothelial cells and multiple layers of smooth
11 muscle cells, representing mature and highly
12 differentiated arteries. Venous lumina are less visible
13 and present as partially collapsed slits. Within the
14 periphery are abundant capillary lumina, and the entire
15 vascular bundle is surrounded by a continuous
16 fibrocellular capsule (Figure 6). Further examination of
17 this resource at higher magnification revealed the
18 relatively rich collagen component of vascular structure
19 as well as the abundance of endothelial cell-lined
20 capillary structures (Figure 12). The presence of two
21 distinct, yet prominent, round structures, marked with
22 asteriks were also observed. These structures displayed
23 histological characteristics of neuronal-like
24 structures. Collectively these data suggest that HBGF-1
25 is capable of signaling a variety of the squamous
26 mesothelial cells of the serosa and the proximal cells of
27 the tunica adventita to initiate angiogenesis. The
28 appearance of mesoderm- and neuroectoderm-derived cells
29 is consistent with the ability of HBGF-1 to act as a
30 mitogen in vitro for epithelial cells, fibroblasts,
31 smooth muscle cells, mesothelial cells, endothelial

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1 cells, astrocytes and oligodendrocytes. The presence of
2 neuronal-like structures is also consistent with the
3 nerve growth factor (NGF)-like biological activity of
4 HBGF-1 to induce neurite extension and survival of PC12
5 cells in vitro.

6 EXAMPLE 5 AND COMPARATIVE EXAMPLE B

7 Example 5 demonstrates that the presence of a
8 large organized solid matrix, containing a network of
9 mature muscular neovessel formations of Example 4 and
10 which are contiguous with the host's vascular tree in
11 situ, permits successful selective cell transplantation.

12 Comparative Example B demonstrates that the use of
13 the same materials and procedures of Example 5 without
14 HBGF-1 did not sustain selective cell transplantation.

15 Homozygous Gunn rats lack
16 UDP-glucuronosyltransferase for bilirubin and cannot
17 efficiently excrete bilirubin. For this reason, Gunn
18 rats exhibit lifelong nonhemolytic unconjugated
19 hyperbilirubinemia. In order to examine the genetic
20 therapy potential of this system, hepatocytes were
21 harvested by collagenase perfusion of syngeneic Wistar
22 (RHA) rats. The Wistar rat is genetically identical to
23 the Gunn rat except that it contains a normal bilirubin
24 conjugation locus.

25 In Example 5, HBGF-1 adsorbed collagen (Type IV)
26 coated PTFE fibers were implanted next to the liver and
27 after ten to fourteen days the peritoneal cavity was

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1 surgically opened revealing numerous neovessel formations
2 both protruding from the liver and extending into the
3 bundle of fibers (Figure 7) and connecting the bowl loop
4 with richly vascular bridges. Primary hepatocytes
5 harvested from syngeneic Wistar (RHA) rats were injected
6 into the fiber network of the vascularized fibers.
7 Immediately, serum bilirubin levels began to decrease and
8 ten days after hepatocyte injections, the serum bilirubin
9 levels had decreased by 50 percent. A gradual decrease
10 to greater than 60 percent was observed for the duration
11 of the experiment (60 days) as shown in Figure 13A.
12 Experiments have determined that reduced levels of serum
13 bilirubin (>60%) can be maintained at least 181 days and
14 histological examination of these long-term implants
15 contain viable hepatocytes. These data suggest that
16 HBGF-1 fiber implant model functions in vivo as a
17 receptacle for the successful site-specific introduction
18 of cells capable of expressing a differentiated
19 physiologic function.

20 In Comparative Example B, the hepatocytes were
21 seeded onto collagen (Type IV) coated PTFE fibers, which
22 did not contain adsorbed HBGF-1, and surgically implanted
23 on the right lobe of the liver. The serum bilirubin
24 levels decreased to approximately 50 percent. This was
25 followed immediately by a sharp reversion to the original
26 serum bilirubin level. Figure 13B shows that the serum
27 bilirubin levels remained constant for the duration of
28 the experiment (60 days). Histological examination of
29 these implants after twenty days suggested that
30 accumulating levels of toxic-like acids within the fiber
31 implant led to the ultimate death of the transplanted
32 hepatocytes.

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1 The long-term HBGF-1 fiber implant model of
2 Example 5 induces a prominent angiotropic and neurotropic
3 response when appropriately implanted in the rat.
4 Example 5 demonstrates the ability of HBGF-1 to induce,
5 sustain, and maintain the anatomical coordination of
6 highly sophisticated and widely diversified mammalian
7 cell types in vivo. The interrelationships between
8 extracellular matrix components and
9 differentiation-specific gene regulation can provide
10 information critical for genetic engineering therapies.
11 This invention may also prove useful as a site-specific
12 transgenic alternative with the ability to understand the
13 temporal and coordinated expression of growth and
14 differentiation signals during neuronal and angiogenic
15 development in the adult.

16 EXAMPLE 6

17 Example 6 demonstrates the neovascular device of
18 this invention wherein genetically engineered cells are
19 seeded into the device. Example 6 is as follows.

A. The construction of the pG2N retroviral vector, that was used to genetically engineer endothelial cells to produce rat growth hormone, was performed with SV40 promoted neomycin resistance gene and a rat growth hormone cDNA. These were placed into the pB2 retroviral vector provided by the Laboratory of Molecular Hematology at NIH. A growth hormone cDNA was obtained by digesting the plasmid RGH-1 according to Nature 270 (1977):494 with

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1 Xho I and Mae II restriction endonucleases from
2 Boehringer Mannheim Biochemicals. This rat growth
3 hormone cDNA was electrophoretically isolated out of an
4 agarose gel and purified via binding/elution to glass
5 beads sold by the tradename, Geneclean Bio, 101, La
6 Jolla, California. This growth hormone cDNA was then
7 blunted using the large fragment of DNA polymerase Klenow
8 known by the name, from New England Biolabs and
9 nucleotide triphosphates as recommended by the
10 manufacturer. This fragment was then purified with
11 Geneclean product.

12 The B2 vector was constructed in order to replace
13 the Neo^R gene in N2 according to M.A. Eglitis, et al.,
14 Science 230 (1985):1395; D. Armentano, et al., J. Virol
15 61 (1987):1647 with a multiple cloning site. N2 was
16 first digested with Eco RI, thereby releasing both the 5'
17 and 3' LTRs with the adjoining MoMLV flanking sequences.
18 The 3' LTR fragment was ligated into the EcoRI site of
19 the plasmid GEM4 from Promega Biotech. The 5' LTR
20 fragment with its flanking gag sequence was then digested
21 with Cla I, Hind III linkers were added, and the fragment
22 was inserted into the Hind III site of pGEM4.

23 The pB2 vector was digested with the HincII
24 restriction endonuclease from New England Biolabs, and
25 phosphatased using calf alkaline phosphatase from
26 Boehringer Mannheim Biochemicals. The pB2 plasmid was
27 then purified with the Geneclean product. The pB2 vector
28 and the rat growth hormone cDNA were then ligated using
29 T4 ligase from New England Biolabs, pG2 was then digested

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1 with BamHI from New England Biolabs, purified with the
2 Geneclean Bio 101 product, and blunt ended with the
3 Klenow fragment. A 340 base pair SV40 promoted neomycin
4 resistance gene fragment was isolated from the pSV2CAT
5 plasmid (ATCC accession number 37155) by digesting with
6 PvuII and HindIII from New England Biolabs. This
7 fragment was isolated by agarose gel electrophoresis and
8 purified with the Geneclean product. The SV40-neomycin
9 resistance fragment was then ligated using T4 ligase from
10 New England Biolabs with pG2 and transformed into DH5
11 competent bacteria per the manufacturer's instructions
12 (BRL). Colonies were screened and the resulting plasmid
13 construct was called pG2N. The SAX vector was obtained
14 as described in Proc. Natl. Acad. Sci. USA 83
15 (1988):6563.

16 The recombinant vectors, N2, SAX, G2N, used in
17 this example were each separately transfected into the
18 currently available retroviral vector packaging cell
19 lines, including the amphotropic packaging lines, PA317
20 Mol. Cell. Biol. 6(1986):2895, and the ecotropic line,
21 Psi2, Cell 33(1983):153. These lines were developed in
22 order to allow the production of helper virus-free
23 retroviral vector particles.

24 B. The CD4 containing plasmid, p4B, which was a gift of
25 Richard Axel of College of Physicians and Surgeons
26 Columbia University, New York, New York, was digested
27 with the restriction endonucleases Eco RI and Bam HI from
28 New England Biolabs, Beverly, Massachusetts, to release
29 the CD4 gene which was isolated by agarose gel

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1 electrophoresis followed by purification via
2 binding/elution to glass beads using the Geneclean
3 product, Bio 101, La Jolla, California, in the manner
4 recommended by the manufacturer. The CD4 fragment was
5 ligated, using T4 DNA ligase as recommended by the
6 supplier, into Eco RI plus Bam HI cut Bluescript cloning
7 vector from Stratagene Co., La Jolla, California. The
8 ligation was then transformed into competent DH5 alpha
9 bacteria from Bethesda Research Labs, Gaithersburg,
10 Maryland, and white colonies were isolated and screened
11 for proper insert size to yield the plasmid pCDW. To
12 produce a suitable plasmid based expression vector for
13 the CD4 gene, the plasmid SV2neo, obtained from American
14 Type Culture Collection, Rockville, Maryland, was
15 digested with Hind III plus Hpa I. A synthetic
16 polylinker sequence from the pUC-13 vector from
17 Pharmacia, Piscataway, New Jersey, was inserted via T4
18 DNA ligase in place of the Neo^R gene of PsV2neo. This
19 ligation was transformed into DH5 bacteria from Bethesda
20 Research Labs and colonies screened for the presence of
21 restriction enzyme sites unique to the polylinker to
22 yield the vector pSVPL. The pSCPL expression vector was
23 further modified by the insertion of an Xho I linker
24 using conditions and reagents suggested and supplied by
25 New England Biolabs, into the Pvu II site on the 5' side
26 of the SV40 early region promoter to produce pSVPLX.

27 The pCDW and pSVPLX plasmids were digested with
28 enzymes Hind III plus Xba I from New England Biolabs and
29 their DNAs isolated using the Geneclean product following
30 agarose gel electrophoresis. Ligation of the CD4

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1 fragment into the pSVPLX vector was performed and
2 colonies were screened to yield pSVCDW in which the SV40
3 virus early region promoter is used to drive the
4 expression of the complete CD4 gene product. The next
5 step was to produce a form of the CD4 gene such that it
6 would be exported from the cell as an extracellular
7 product.

8 C. The production of a soluble form of CD4 was
9 accomplished by the use of a specially designed
10 oligonucleotide adaptor to produce a mutant form of the
11 CD4 gene. This adaptor has the unique property that when
12 inserted into the Nhe I site of the CD4 gene it produces
13 the precise premature termination of the CD4 protein
14 amino acid sequence while regenerating the Nhe I site and
15 creating a new Hpa I site. This oligonucleotide adaptor,
16 synthesized by Midland Certified Reagent Co., was
17 produced by annealing two phosphorylated
18 oligonucleotides: (1) 5'CTAGCITGAGTGAGIT 3' and (2)
19 AACTCACTCAAG. This product was then ligated into the
20 site of pSVCDW. The ligation reaction was then cleaved
21 with Hpa I and then Xho I linkers were added. The linker
22 reaction was terminated by heating at 65°C for 15 minutes
23 and then subjected to digestion with Xho I restriction
24 endonuclease from New England Biolabs. This reaction was
25 then subjected to agarose gel electrophoresis and the
26 fragment containing the SV40-CD4 adaptor isolated using
27 the Geneclean product. The retroviral vector N2 was
28 prepared to accept the SV40-CD4-adaptor fragment by
29 digestion with Xho I and treatment with calf intestinal
30 phosphatase from Boehringer Mannheim, Indianapolis,
31 Indiana.

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1 The ligation of a CD4 expression cassette was performed
2 with an insert to vector ratio of 5:1 and then
3 transformed in DH5 competent bacteria from Bethesda
4 Research Labs. Constructs were analyzed by restriction
5 endonuclease digestion to screen for orientation and then
6 grow up in large scale. The construct where the SV40
7 virus promoter is in the same orientation as the viral
8 LTR promoters is known as SSC while the construction in
9 the reverse orientation is called SCSX.

10 The SSC vector is packaged into PA 317 cell line
11 as described by Miller, et al., supra, to provide PA 317
12 cells capable of producing soluble CD4 protein. The SSC
13 vector packaged PA 317 cells were used to transduce
14 rabbit endothelial cells as described above. The
15 transduced endothelial cells expressed soluble CD4.

16 D. Collagen sponges containing adsorbed HBGF-1 of the
17 type previously described were surgically implanted in
18 the abdominal cavity of a rat near the liver. Sponges
19 were surgically removed seven to ten days
20 post-implantation and digested 30 to 60 minutes at 27°C
21 with a solution of collagenase in phosphate buffered
22 saline in a concentration of 1mg/ml using a tissue
23 culture incubator at 5 percent in CO₂. Released cells
24 were collected by centrifugation for 10 minutes at 1000
25 RPM at 20°C. The cells were washed once with phosphate
26 buffered saline (PBS) and pelleted by centrifugation.
27 Cells were resuspended with two volumes of 30 ml of media
28 containing: M199 media (Gibco); ECGF (crude brain
29 extract) 7.2mg; Heparin (Upjohn) 750 units;

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1 and 20 percent conditioned cellular media collected as
2 supernatant from confluent dishes after 48 hours of
3 either bovine aortic or human umbilical vein endothelial
4 cells. The other media contained: 10 percent fetal calf
5 serum (Hyclone); 3000 units Penicillan G (Biofluids); and
6 3000 units streptomycin sulfate (Biofluids) and the cells
7 were plated for 16 hours on 100 mm tissue culture disk
8 coated with fibronectin (human) using $1\mu\text{g}/\text{cm}^2$. Plated
9 cells were washed with PBS three times and fed 15ml of
10 previously mentioned media. Media was changed every 2
11 days for the duration of the procedures.

12 Selected rat endothelial cells were transduced
13 with N-7, SAX, G2N and SSC vectors by the following
14 procedures:

15 1. 2×10^6 microendothelial cells (monolayer 80
16 percent confluent)

17 2. 2×10^6 cfu/ml viral supernatant

18 3. Polybrene ($8\mu\text{g}/\text{ml}$)

19 - Combine 1, 2, 3 in 5 ml total volume for 2-3
20 hours at 37°C (5 percent CO_2).

21 - Add 20ml of tissue culture media for 16 hours,
22 at 37°C (5 percent CO_2).

23 - Aspirate off media (virus containing), add
24 fresh culture media.

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1 - After 48-96 hours, add G418 (800ug/ml) and
2 culture media.

3 - Select for one to two weeks changing media
4 every two days.

5 The following are procedures for seeding a sponge
6 with the transduced endothelial cells described above.

7 A. The endothelial cells are seeded directly onto a
8 HBGF-1 adsorbed, collagen coated PTFE fiber sponge, and
9 the sponge is implanted back into the same animal used as
10 the source of endothelial cells. The site of
11 implantation can be subcutaneous, intraperitoneal, or at
12 or near the site of the organ that normally produces the
13 new product encoded by the gene transduced into the
14 endothelial cells. The sponge implant generates its own
15 vascularization within 5 to 10 days, as described in
16 earlier examples. The engineered endothelial cells are
17 maintained on the implant such that the new gene product
18 is delivered directly into the circulation after
19 secretion from the cell. The production of the gene
20 product is monitored either by direct measurement of its
21 serum levels, by the biochemical or physiological effect
22 of the agent, or both.

23 B. An HBGF-1 adsorbed, collagen coated PTFE fiber sponge
24 is preimplanted at the desired site, as described above,
25 and at the time determined to be optional for that
26 implant site for establishment of neovascularization.
27 The transformed cells are injected directly into the

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1 already-vascularized fiber sponge. The advantage of this
2 method is that the engineered cells are more rapidly and
3 effectively established in the implant or migrate back
4 into the parent organ (e.g., liver). The product begins
5 to enter the circulation much sooner than with method A
6 above. Production of the new gene product is measured as
7 described in method A. This procedure can be applied to
8 a number of different cell types capable of being
9 sampled, genetically engineered in vivo, and reinserted
10 via the fiber sponge implant. Such cells include
11 fibroblasts, hepatocytes, smooth muscle cells, bone
12 marrow cells and others. The products delivered to the
13 circulation can be any peptide or protein whose gene can
14 be inserted into a cell and whose product is desired to
15 be delivered.

16

EXAMPLE 7

17 Gortex shunt tubes were surgically implanted into
18 the peritoneum of rats, in such a way as to form a loop,
19 with each end contacting the aorta. The tubes contained
20 either a Gelfoam (Collagen I) sponge impregnated with
21 HBGF-1 (1 ng/ml) or a bundle of "angel hair" Gortex
22 fibers, coated with Collagen I and impregnated with
23 HBGF-1 (1 ng/ml). The tubes were left in the animals for
24 one month, then surgically extracted, grossly examined
25 for blood vessel formation, and the sponge prepared for
26 histological examination. As shown in Figure 14A, the

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1 tube that had contained the Gelfoam sponge contained no
2 new blood vessels, and the sponge had completely
3 dissolved. In contrast, the angel-hair Gortex fiber
4 bundles became significantly vascularized (Figure 14B),
5 with higher magnification showing the capillary
6 structures (Figures 14C, D).

7 This experiment provides an example of directing
8 neovascularization to a particular site, with a two
9 component device. The first component, a tube or pouch,
10 can provide a receptacle in which implanted cells,
11 genetically engineered or normal, can be seeded. It is
12 possible that such a site may be immunologically
13 privileged, and allow cells from another individual, or
14 even another species, to survive and produce a desired
15 product.

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1 WHAT IS CLAIMED IS

2 1. A neovascularization device comprising
3 a biocompatible support; and
4 a biological response modifier for inducing
5 neovascularization, said biological response modifier
6 being adsorbed to said biochemical support.

7 2. The neovascularization device of claim 1
8 wherein said biocompatible support is an absorbable
9 support.

10 3. The neovascularization device of claim 2
11 further comprising:

12 a non-absorbable support.

13 4. The neovascularization device of claim 1
14 wherein said biocompatible support is a non-absorbable
15 support.

16 5. The neovascularization device of claim 2
17 wherein said absorbable support is a member selected from
18 the group consisting of collagen, laminin, fibronectins,
19 gelatin, glycosaminoglycan, glycoproteins, proteoglycans
20 and mixtures thereof.

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1 6. The neovascularization device of claim 1
2 wherein said biological response modifier is a member
3 selected from the group consisting of a hormone, a
4 hormone prototype, a hydrolase, and mixtures thereof.

5 7. The neovascularization device of claim 6
6 wherein said hormone is an angiogenic and neurotrophic
7 growth factor being a member selected from the group
8 consisting of HBGF-I, HBGF-II, an HBGF-I prototype, an
9 HBGF-II prototype, and mixtures thereof.

10 8. The neovascularization device of claim 6
11 wherein said hydrolase is heparinase, collagenase,
12 plasmin, a plasminogen activator, thrombin, heparatinase,
13 and mixtures thereof.

14 9. The neovascularization device of claim 1
15 wherein said biological response modifier is an
16 angiogenic growth factor, said angiogenic growth factor
17 being in a concentration of about 1 to about 10 nanograms
18 per mm³ of said support.

19 10. The neovascularization device of claim 3
20 wherein said non-absorbable support is a member selected
21 from the group consisting of nylon, rayon, dacron,
22 polypropylene, polyethylene, PTFE, collagen I, collagen
23 IV, kerratin, and glycolipid.

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1 11. The neovascularization device of claim 4
2 wherein said non-absorbable support is a member selected
3 from the group consisting of nylon, rayon, dacron,
4 polypropylene, polyethylene, PTFE, collagen I, collagen
5 IV, kerratin, and glycolipid.

6 12. The neovascularization device of claim 2
7 wherein said absorbable support is gelatin.

8 13. A neovascularization device comprising:
9 an absorbable support;

10 a non-absorbable support, said absorbable support
11 being adsorbed to said non-absorbable support; and

12 a biological response modifier in sufficient
13 concentration for inducing in vivo site directed
14 neovascularization, said biological response modifier
15 being adsorbed to said absorbable support.

16 14. The neovascularization device of claim 13
17 wherein said absorbable support is a member selected from
18 the group consisting of collagen, laminin, fibronectins,
19 gelatin, glycosaminoglycan, glycoproteins, proteoglycans
20 and mixtures thereof.

21 15. The neovascularization device of claim 13
22 wherein said biological response modifier is a member
23 selected from the group consisting of a hormone, a
24 hormone prototype, a hydrolase, and mixtures thereof.

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1 16. The neovascularization device of claim 15
2 wherein said hormone is an angiogenic and neurotrophic
3 growth factor being a member selected from the group
4 consisting of HBGF-I, HBGF-II, an HBGF-I prototype, an
5 HBGF-II prototype, and mixtures thereof.

6 17. The neovascularization device of claim 15
7 wherein said hydrolase is heparinase, collagenase,
8 plasmin, a plasminogen activator, thrombin, heparatinase,
9 and mixtures thereof.

10 18. The neovascularization device of claim 13
11 wherein said biological response modifier is an
12 angiogenic growth factor, said angiogenic growth factor
13 being in a concentration of about 1 to about 10 nanograms
14 per mm³ of said per mm³ of both said absorbable support
15 and non-absorbable support.

16 19. The neovascularization device of claim 13
17 wherein said non-absorbable support is a member selected
18 from the group consisting of nylon, rayon, dacron,
19 polypropylene, polyethylene, PTFE, collagen I, collagen
20 IV, kerratin, and glycolipid.

21 20. A neovascularization device comprising:
22 a biocompatible support; and

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1 25. The neovascularization device of claim 20
2 wherein said hormone is an angiogenic and neurotrophic
3 growth factor being a member selected from the group
4 consisting of HBGF-I, HBGF-II, an HBGF-I prototype, an
5 HBGF-II prototype, and mixtures thereof.

6 26. The neovascularization device of claim 20
7 wherein said hydrolase is heparinase, collagenase,
8 plasmin, a plasminogen activator, thrombin, heparatinase,
9 and mixtures thereof.

10 27. The neovascularization device of claim 22
11 wherein said support is a member selected from the group
12 consisting of nylon, rayon, dacron, polypropylene,
13 polyethylene, PTFE, collagen I, collagen IV, kerratin,
14 and glycolipid.

15 28. The neovascularization device of claim 23
16 wherein said non-absorbable support is a member selected
17 from the group consisting of nylon, rayon, dacron,
18 polypropylene, polyethylene, PTFE, collagen I, collagen
19 IV, kerratin, and glycolipid.

20 29. A process for producing neovascularization
21 comprising:

22 adsorbing a biological response modifier for
23 inducing neovascularization onto a biocompatible support;

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1 contacting a therapeutically effective amount of
2 said adsorbed biological response modifier to at least
3 one selected tissue in an organism; and

4 directing in vivo growth of neovascular cells at
5 said contacted, selected tissue for a sufficient time to
6 obtain a vascular structure.

7 30. The process for producing neovascularization
8 of claim 29 wherein said neovascular cells contain a
9 genetic insert.

10 31. The process for producing neovascularization
11 of claim 30 wherein said genetic insert enables said
12 neovascular cells to secrete a biological product.

13 32. The process for producing neovascularization
14 of claim 31 wherein said biological product is a
15 biological response modifier.

16 33. The process for producing neovascularization
17 of claim 32 wherein said biological response modifier is
18 a member selected from the group consisting of a hormone,
19 a hormone precursor, and a hydrolase.

20 34. The process for producing neovascularization
21 of claim 29 further comprising:

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1 seeding said vascular structure with non-vascular
2 cells.

3 35. The process for producing neovascularization
4 of claim 34 wherein said seeded cells secrete a desired
5 biological product.

6 36. The process for producing neovascularization
7 of claim 34 wherein said seeded cells perform a desired
8 metabolic function.

9 37. The process for producing neovascularization
10 of claim 29 wherein said biocompatible support is an
11 absorbable support.

12 38. The neovascularization device of claim 37
13 further comprising:

14 a non-absorbable support.

15 39. The neovascularization device of claim 29
16 wherein said biocompatible support is a non-absorbable
17 support.

18 40. The neovascularization device of claim 37
19 wherein said absorbable support is a member selected from
20 the group consisting of collagen, laminin, fibronectins,
21 gelatin, glycosaminoglycan, glycoproteins, proteoglycans
22 and mixtures thereof.

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1 41. The neovascularization device of claim 29
2 wherein said biological response modifier is a member
3 selected from the group consisting of a hormone, a
4 hormone prototype, a hydrolase, and mixtures thereof.

5 42. The neovascularization device of claim 41
6 wherein said hormone is an angiogenic and neurotrophic
7 growth factor being a member selected from the group
8 consisting of HBGF-I, HBGF-II, an HBGF-I prototype, an
9 HBGF-II prototype, and mixtures thereof.

10 43. The neovascularization device of claim 41
11 wherein said hydrolase is heparinase, collagenase,
12 plasmin, a plasminogen activator, thrombin, heparatinase,
13 and mixtures thereof.

14 44. The neovascularization device of claim 29
15 wherein said biological response modifier is an
16 angiogenic growth factor, said angiogenic growth factor
17 being in a concentration of about 1 to about 10 nanograms
18 per mm³ of said support.

19 45. The neovascularization device of claim 38
20 wherein said non-absorbable support is a member selected
21 from the group consisting of nylon, rayon, dacron,
22 polypropylene, polyethylene, PTFE, collagen I, collagen
23 IV, kerratin, and glycolipid.

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1 46. The neovascularization device of claim 39
2 wherein said non-absorbable support is a member selected
3 from the group consisting of nylon, rayon, dacron,
4 polypropylene, polyethylene, PTFE, collagen I, collagen
5 IV, kerratin, and glycolipid.

6 47. A product for promoting neovascularization,
7 comprising:

8 a support including an extracellular matrix
9 protein and a biological response modifier.

10 48. The product of claim 47 wherein the support
11 includes cells capable of expressing a metabolite whereby
12 the product is capable of inducing organoid
13 neovascularization.

14 49. The product of claim 48 wherein the cells are
15 genetically engineered to express a heterologous protein.

16 50. The product of claim 49 wherein the support
17 is a non-absorbable support.

18 51. The product of claim 50 wherein the
19 biological response modifier is absorbed to the
20 extracellular matrix protein included in the
21 non-absorbable support.

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1 52. The product of claim 51 wherein said
2 biological response modifier is a member selected from
3 the group consisting of a hormone, a hormone prototype, a
4 hydrolase, and mixtures thereof.

5 53. The product of claim 52 wherein the
6 biological response modifier is at least one member
7 selected from the group consisting of heparinase,
8 collagenase, plasmin, a plasminogen activator, thrombin,
9 and heparatinase.

10 54. The product of claim 52 wherein the
11 biological response modifier is at least one member
12 selected from the group consisting of HBGF-I, HBGF-II,
13 and HBGF-I prototype, and an HBGF-II prototype.

14 55. The product of claim 51 wherein said
15 biological response modifier is an angiogenic growth
16 factor, said angiogenic growth factor being in a
17 concentration of about 1 to about 10 nanograms per mm³ of
18 said support.

19 56. The product of claim 51 wherein said
20 non-adsorbable support is a member selected from the
21 group consisting of nylon, rayon, dacron, polypropylene,
22 polyethylene, PTFE, and cross-linked collagen IV.

23 57. The product of claim 51 wherein the
24 extracellular matrix protein is at least one member
25 selected from the group consisting of collagen, laminin,
26 fibronectins, gelatin, glycosaminoglycan, glycoproteins,
27 and proteoglycans.

1 / 14

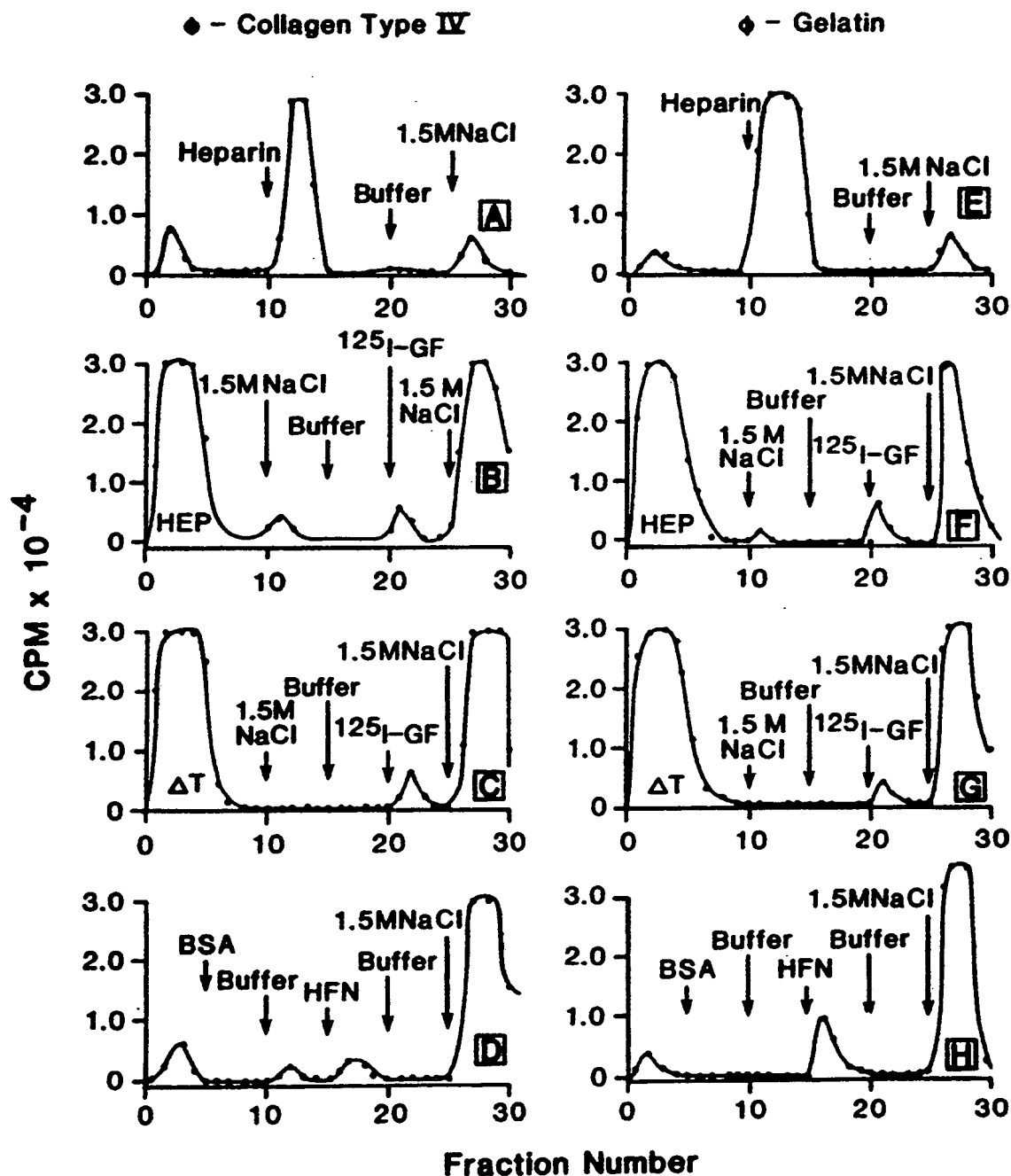


FIG. I

SUBSTITUTE SHEET

2 / 14

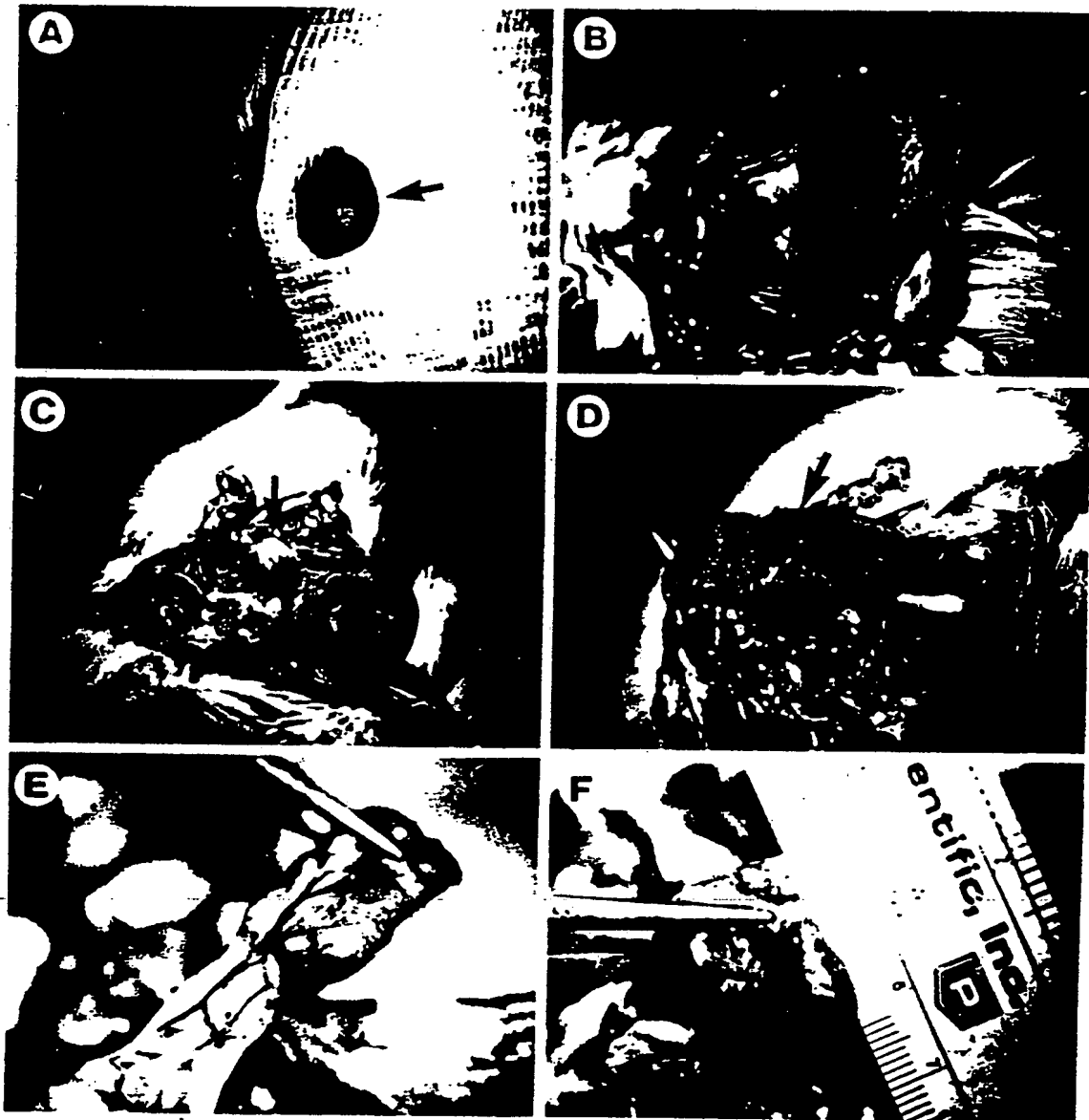


FIG. 2

3 / 14

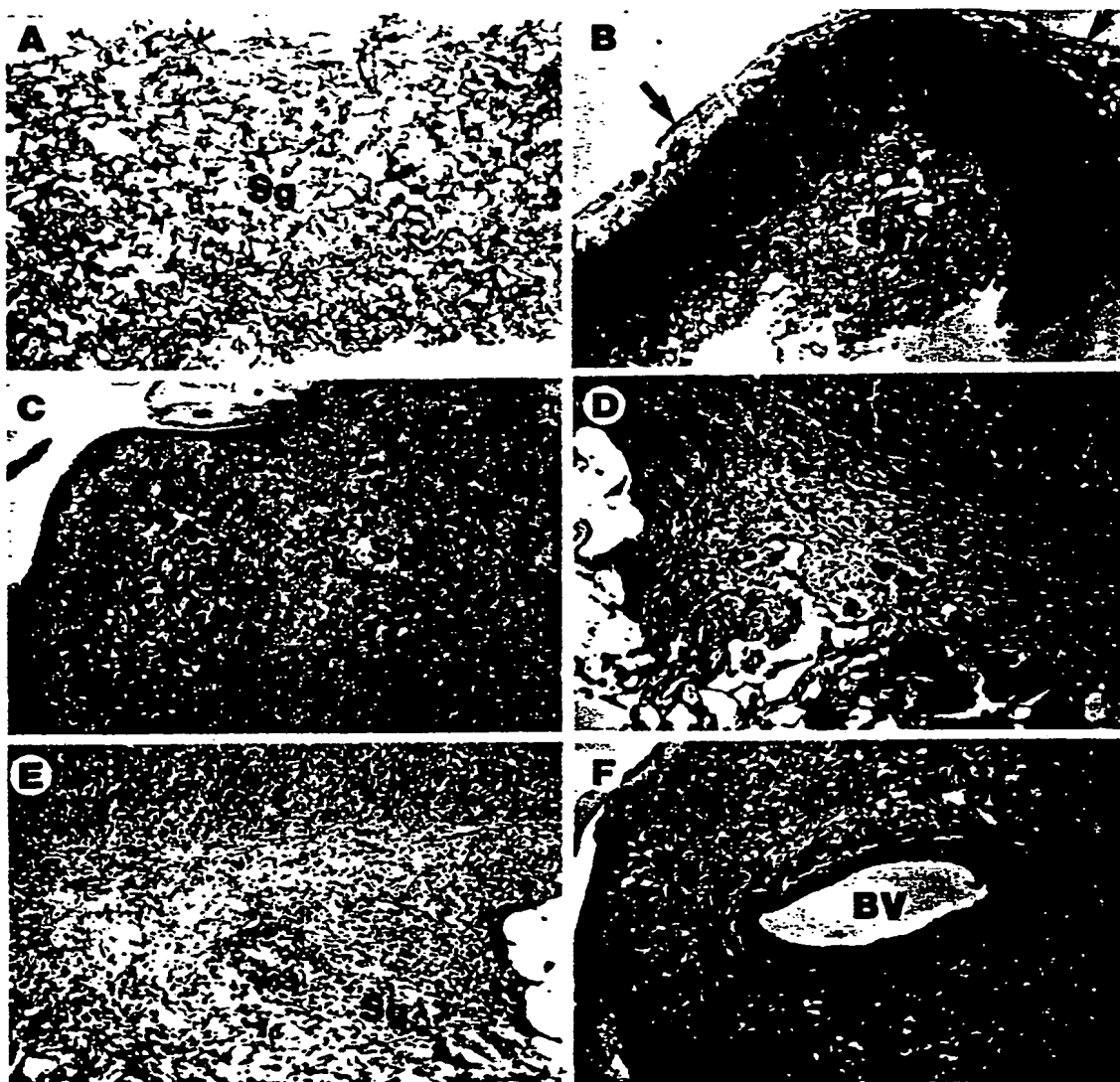


FIG. 3

SUBSTITUTE SHEET

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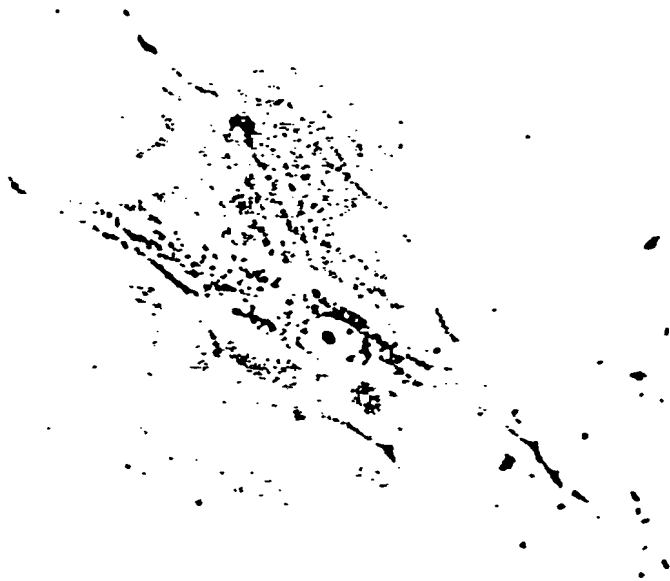


FIG. 4

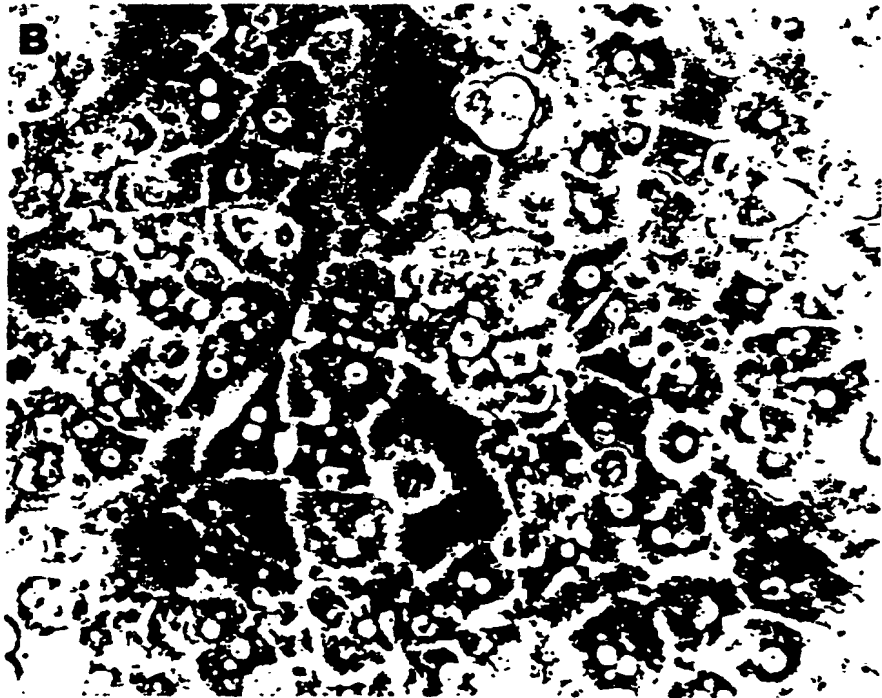
5 / 14

FIG. 5

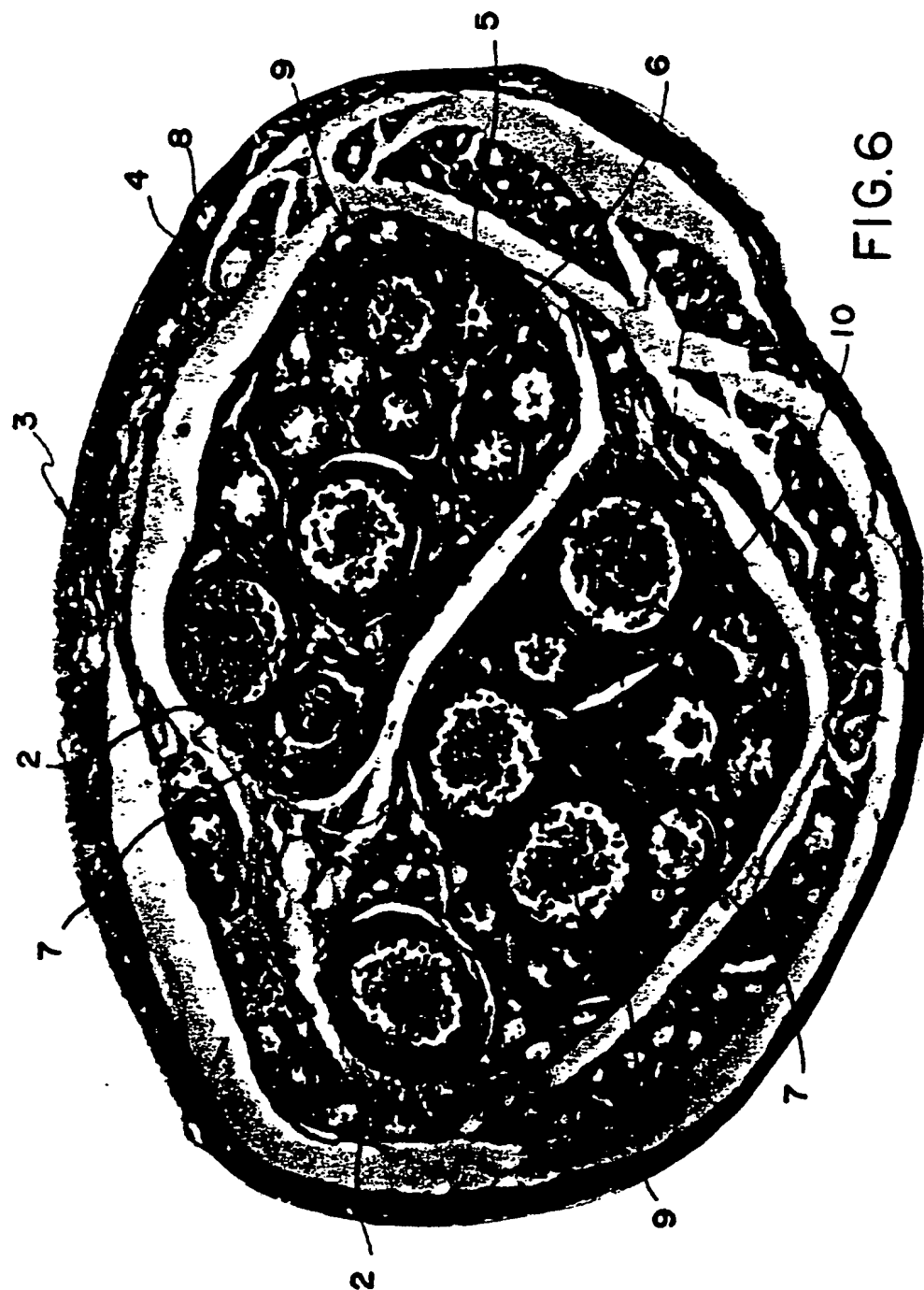
A



B



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SUBSTITUTE SHEET

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FIG. 7

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FIG. 8

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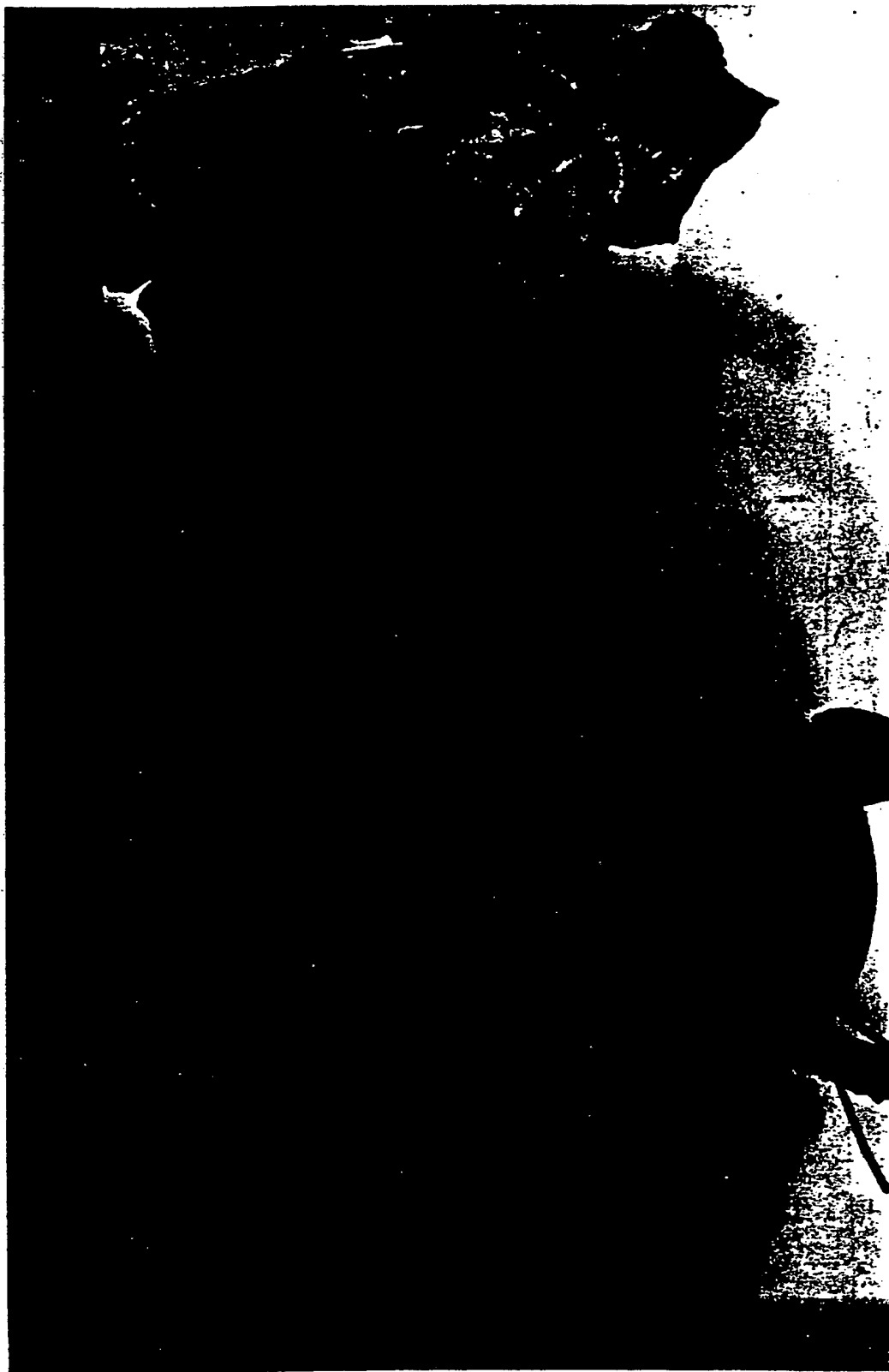


FIG. 9

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FIG. 10

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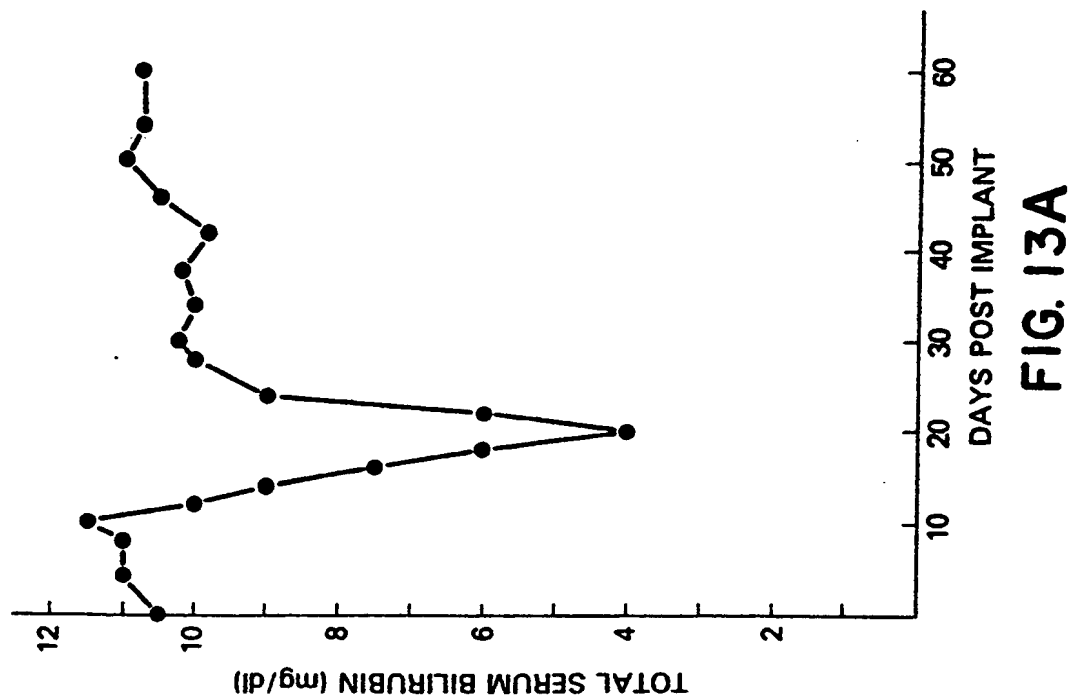
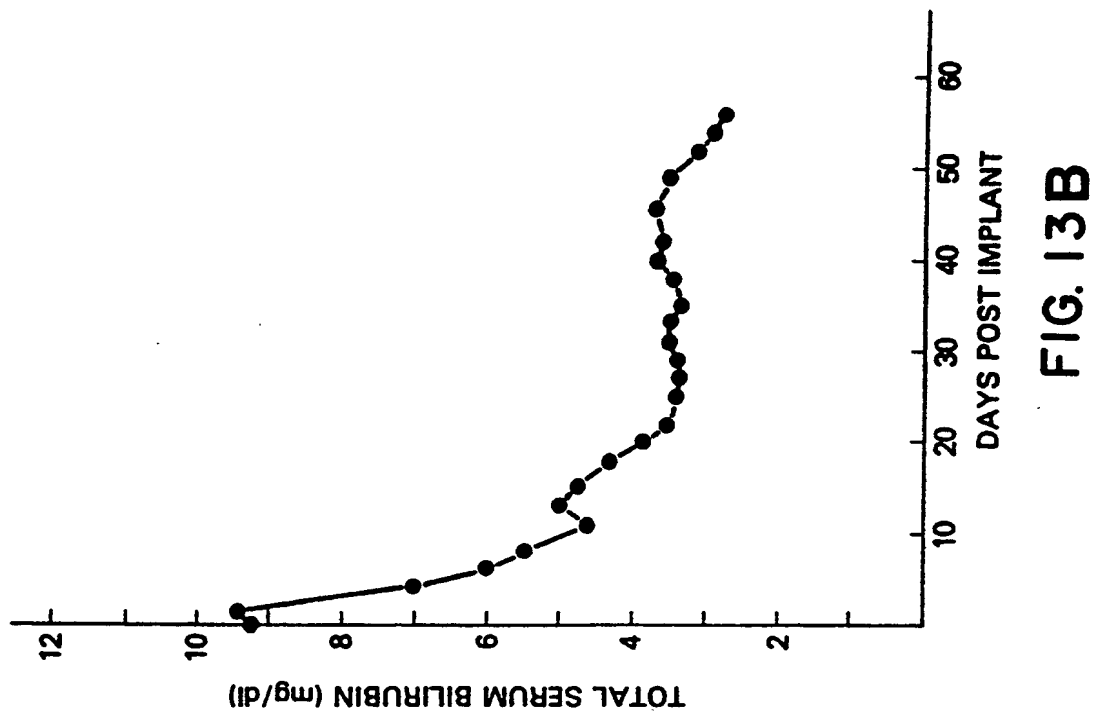
FIG. 11

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FIG. 12

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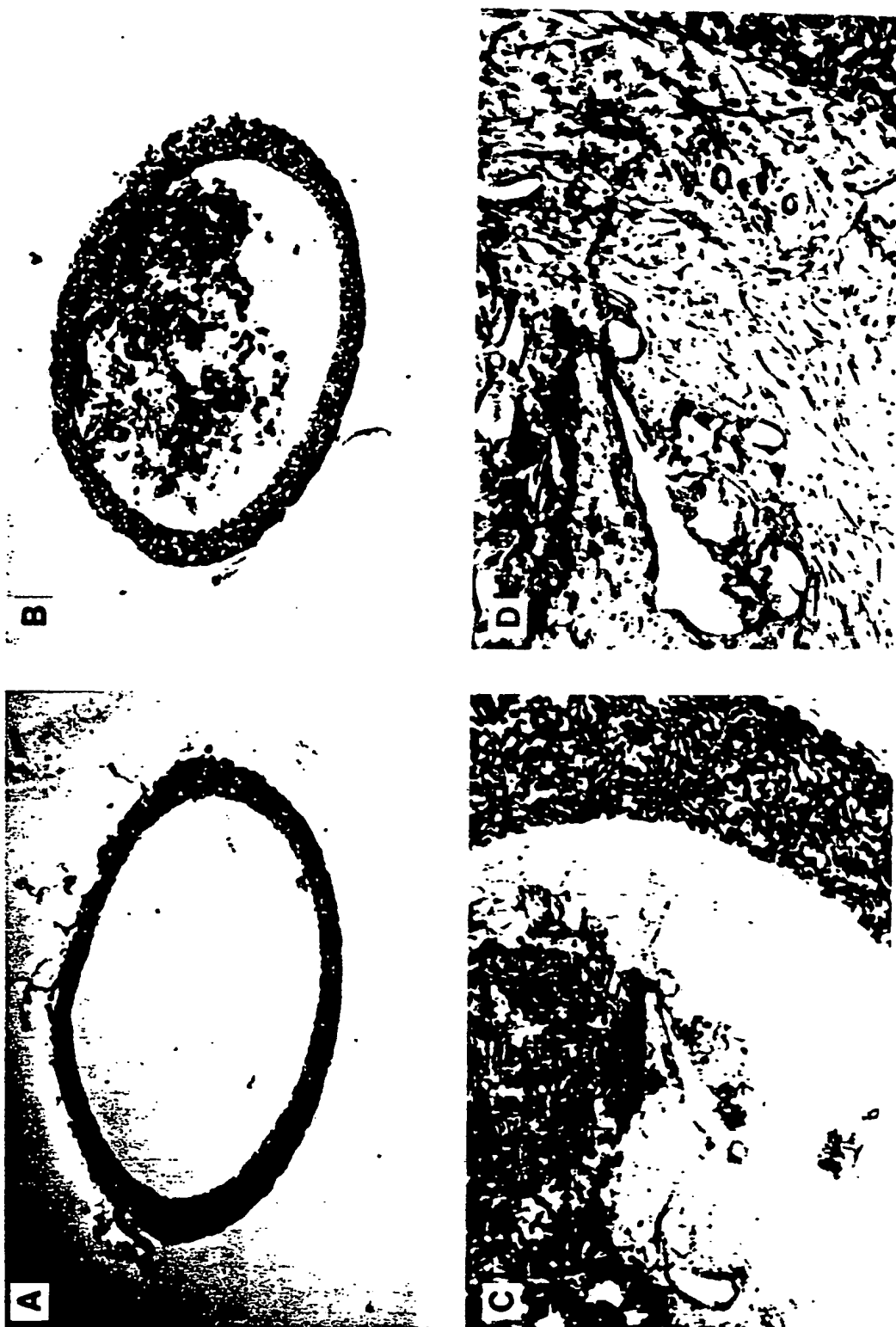


FIG. 14

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/00742

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC IPC(4): A61K 37/02, 37/24, 37/54; C12N 5/00, 11/02, 11/08 U.S./Cl.: 514/2, 8; 424/94.6, 94.61, 94.63, 94.64; 435/177, 180, 240.23		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
U.S.	424/94.6, 94.61, 94.63, 94.64; 435/177, 180, 240.23 514/2, 8, 21, 774, 801	
Documentation Searched other than Minimum Documentation to the extent that such documents are included in the fields searched ⁸ DATABASES: CHEMICAL ABSTRACTS SERVICES ONLINE (FILE CA, 1967-1989; FILE BIOSIS, 1969-1989). See Attachment for Search terms.		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
Y	US, A, 4,699,141 (Lamberton et al) 13 October 1987, See Entire Document	13-16, 18, 19, 22, 27, 30-38, 40, 42, 45, 47-52, 54-57
X	US, A, 4,699,141 (Lamberton et al) 13 October 1987, See Entire Document	1, 4, 29, 34, 35, 36, 39, 47, 48
Y	British Journal of Experimental Pathology Volume 68, Issued 1987, Andrade et al, "Quantitative in Vivo Studies on angiogenesis in a rat sponge model", pages 755-766, Entire Document.	1, 4, 6, 9-11, 20, 23, 28, 29, 39, 41, 44, 46
Y	Proceedings of the National Academy of Sciences, USA, Volume 82, Issued November 1985, Buckley et al, "Sustained release of epidermal growth factor accelerates wound repair," pages 7340-7344, Entire Document.	1, 4, 6, 9, 11, 20, 23, 28, 29, 39, 41, 44, 45, 46
<div style="display: flex; justify-content: space-between;"> <div> <p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"Δ" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search 09 May 1989		Date of Mailing of this International Search Report 05 JUL 1989
International Searching Authority ISA/US		Signature of Authorized Officer Gail F. Knox <i>Gail F. Knox</i>

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X	Biochemical and Biophysical Research	1-7,
Y	Communications Volume 147, Issued 15	12-16,
	September 1987 Hayek et al, "An In Vivo model	29,
	for study of the angiogenic effects of basic	37-42
	fibroblast growth factor", pages 876-880,	9-11,
	Entire document.	18-25,
		27-28,
		44-46

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter^{1,2} not required to be searched by this Authority, namely:

2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out^{1,2}, specifically:

3. ☐ Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No ¹⁸
Y	WO, A 8701728 (Biotechnology Research Partners, LTD) 26 March 1987, pages 1-64, See pages 1-3, 12-16.	1,4,6, 7,9,11, 20,23, 25,28, 29,39, 44,46
X,P	Journal of Cell Biology, Volume 107 (6 part 3), Issued 09 December 1988, Maciag et al, "Heparin-Binding Growth Factor-I (HBGF-I) Bound to Gelatin Induces Site-Specific Neo-vessel Formation In Vivo", Abstract No. 2697, page 479a.	1-57
Y	Surgical Science Series, Volume 2, Issued 1984, Jackson et al, "Effect of Angiogenic Factors on the Vascularization of IVALON Sponge Implants", pages 190-201, See entire document.	1,4,6, 7,9, 11,20, 23,25, 28,29 39,44, 46
Y	Biochemical and Biophysical Research Communications, Volume 142, Issued 30 January 1987, Baird et al, "Fibroblast Growth Factors are Present in the Extracellular Matrix Produced by Endothelial Cells <u>In Vitro</u> . Implications for a Role of heparinase-like Enzymes in the Neovascular Response, pages 428-435, especially page 433.	8,17, 26,43, 53
Y	Cancer Research, Volume 43, Issued June 1983, Vlodavsky et al, "Lymphoma Cell-Mediated Degradation of sulfated Proteoglycans in the Subendothelial Extracellular Matrix: Relationship to Tumor Cell Metastasis", pages 2704-2711, especially page 2710.	8,17, 26,43, 53
Y	Chemical Abstracts, Volume 109, Issued 10 October 1988, Rifkin, "Endothelial Cell proteases and Cellular invasion", Abstract No. 123103	8,17, 26,43 53

Attachment to Form PCT/ISA/210
Part II. FIELDS SEARCHED SEARCH TERMS:

Angiogenesis
Neovascularization
IVALON
Neovessel
formation
sponge
support
biocompatible
transplant
heparinase
heparitinase
collagenase
plasminogen
activator
plasmin
hydrolase